**University of Alberta**

*Global and gene-specific regulation of chromatin metabolism in Saccharomyces cerevisiae*

by

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requirements for the degree of *Doctor of Philosophy*

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For my parents…

**Abstract:**

Chromatin plays an essential role in the regulation of DNA replication, recombination, repair, and transcription. The deposition, remodeling, and modification of nucleosomes are mediated by a diverse group of factors, and chromatin structure and function are subject to physiological regulation. To gain further insights into chromatin regulation, we examined the global and gene-specific regulation of chromatin metabolism in *Saccharomyces cerevisiae*.

This thesis is divided into two parts. Part I provides evidence that dynamic gene targeting of conserved nucleosome assembly factor Asf1 is an important step in chromatin remodeling associated with transcriptional reprogramming. Transcriptional repression involves direct association of Asf1 with a DNA damage response gene involved in dNTP synthesis, *RNR3*. Asf1 promotes the assembly/stabilization of promoter nucleosomes that inhibit transcription. DNA damage signals trigger release of Asf1 from *RNR3* concomitant with chromatin remodeling and transcriptional induction. This work advances the current understanding of histone chaperone function in vivo, and has uncovered a new step in transcriptional regulation by DNA damage signals.

Gene-specific chromatin structure and transcriptional regulation often involve the coordinated action of multiple proteins. It is shown here that other chromatin factors participate in targeting of Asf1 to *RNR3*. These include the histone H2A variant, Htz1, the transcription initiation factor subunit, Bdf1, and the chromatin remodeling enzyme, Isw2. Like Asf1, the function of both Htz1 and Isw2 at *RNR3* is controlled by DNA damage signals. These results provide insight into the molecular identity of the

machinery with which gene-targeted Asf1 functions in the context of transcriptional regulation.

Part II demonstrates a distinct mechanism of chromatin regulation involving the anaphase promoting complex (APC), a conserved protein ubiquitin ligase involved in cell cycle regulation. It is demonstrated that physiological regulation of global histone H3/H4 acetylation by the APC occurs during cell cycle exit into $G_0$. The phosphorylation state of H3 is controlled by the APC in both cycling and $G_0$ cells by a mechanism involving the regulation of both a conserved kinase and a phosphatase. APC mutations perturb both the reconfiguration of histone modification state and normal reprogramming of transcription of cells executing the $G_0$ program in response to nutrient withdrawal.

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**List of Abbreviations:**

| | |
|---|---|
| APC | anaphase promoting complex |
| Asf1 | anti-silencing factor 1 |
| ATP | adenosine-triphosphate |
| βME | β-mercaptoethanol |
| bp | base pair |
| CAF | chromatin assembly factor |
| ChIP | chromatin immunoprecipitation |
| Chip | DNA microarray chip |
| D box | destruction box |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleotide-triphosphate |
| DSB | double strand break |
| EDTA | ethylenediaminetetraacetic acid |
| EtOH | ethanol |
| E1 | ubiquitin activating enzyme |
| E2 | ubiquitin conjugating enzyme |
| E3 | ubiquitin ligase |
| GST | glutathione-*S*-transferase |
| $G_0$ | stationary phase |
| HAT | histone acetyltransferase |
| HDAC | histone deacetylase |
| HFD | histone fold domain |
| HU | hydroxyurea |
| IAA | isoamyl alcohol |
| kDa | kiloDalton |
| MMS | methyl methanesulfonate |
| MNase | Micrococcal Nuclease |
| OD | optical density |
| ORF | open reading frame |
| PAGE | polyacrylamide gel electrophoresis |

| | |
|---|---|
| PCNA | proliferating cell nuclear antigen |
| PCR | polymerase chain reaction |
| PMSF | phenylmethylsulfonyl fluoride |
| rDNA | ribosomal DNA |
| RNA | ribonucleic acid |
| $RNAP_{II}$ | RNA polymerase II |
| Rnr | Ribonucleotide reductase |
| RT | room temperature |
| SDS | sodium dodecyl sulfate |
| $TAF_{II}$ | TBP-associated factor |
| TBP | TATA-binding protein |
| TE | 10 mM Tris, 1 mM EDTA |
| Tris | tris(hydroxymethyl)aminomethane |
| UV | ultraviolet |
| YPD | yeast extract-peptone-2% dextrose |

# Chapter 1:

# Introduction

**Introduction:**

This thesis is divided into two distinct sections. The theme unifying these sections is the regulation of chromatin in budding yeast, *Saccharomyces cerevisiae.*

**Chromatin**

Chromatin is a nuclear mass of genetic material consisting of DNA and proteins. The fundamental repeating unit of chromatin is the nucleosome, comprised of 146 bp of DNA wrapped around a histone octamer core. The assembly of DNA into chromatin begins immediately following replication and is essential for eukaryotic genome maintenance and inheritance, and the nuclear processes of DNA replication, transcription, recombination, and repair are all impacted by the structure and dynamics of chromatin (reviewed in Morales et al., 2001). The process of nucleosome assembly is initiated by deposition of a tetramer of histones H3 and H4 followed by incorporation of two H2A/H2B heterodimers (Smith and Stillman, 1991). Chromatin structure is altered by post-translational histone modification including histone acetylation and deacetylation by histone acetyltransferases (HATs) and deacetylases (HDACs), ATP-dependent chromatin remodeling, and histone replacement (reviewed in Ehrenhofer-Murray, 2004; Kamakaka and Biggins, 2005).

**Part I: Functional regulation of chromatin and transcription**

**Chromatin assembly and remodeling**

The formation of a functional eukaryotic genome depends critically on the process of chromatin assembly. The assembly of nucleosomes from DNA and histones requires a group of proteins called chromatin assembly factors (CAFs) that act in three general assembly pathways, one coupled to DNA replication, one that occurs independently of replication, and one that is coupled to DNA repair (Adams and Kamakaka, 1999; Kornberg and Lorch, 1999; Verreault, 2000; Kadam and Emerson, 2002). Chromatin assembly mechanisms function to create regular arrays of nucleosomes on newly synthesized DNA or to restore chromatin structure at regions where it has been disrupted by alternative chromatin remodeling complexes, transcription, or other processes (Tyler, 2002). Replication-coupled assembly during S phase deposits nucleosomes following passage of the DNA replication fork.

Replication-independent assembly is thought to function to replace nucleosomes lost as a result of histone degradation or displacement during $G_2$, M, and $G_1$. It is expected to play a role in chromatin remodeling events occurring outside of S phase. Repair-coupled chromatin assembly deposits nucleosomes following processing and repair at the site of DNA damage. The functions performed by chromatin assembly factors are intimately linked to histone metabolism. They include formation of distinct chromatin structures, nuclear transport, post-translational modification, and chromatin remodeling (reviewed in Loyola and Almouzni, 2004). In the cell, chromatin assembly is mediated by two classes of factors, the histone chaperones and the ATP-dependent chromatin remodeling factors.

**Histone Chaperones**

Histone chaperones are CAFs which directly bind to the core histones. Anti-silencing factor 1 (Asf1) is an evolutionarily conserved histone chaperone with orthologs in fly, nematode, mouse and human. Asf1 is a central player in many chromatin related processes, including assembly, transcription, silencing, remodeling and repair. Originally discovered as an S phase-specific gene that antagonizes silencing at the mating type loci and telomeres when overexpressed (Le et al., 1997; Singer et al., 1998), it was subsequently found to serve as a histone chaperone that participates in replication-dependent nucleosome assembly through the deposition of histones H3 and H4 tetramers onto naked DNA (Tyler et al., 1999). Asf1 functions in concert with other chromatin regulators such as CAF-1 (Tyler et al., 2001) and Hir proteins (Sharp et al., 2001; Sutton et al., 2001) to promote chromatin assembly following DNA replication and repair (Fig. 1.1). Although Asf1 was originally biochemically purified in complex with newly synthesized histones possessing a characteristic acetylation pattern (Tyler et al., 1999), results from more recent experiments show that the histone binding and deposition activity of Asf1 is independent of acetylation state (Sharp et al., 2001), and support a role for Asf1 in replication-independent chromatin assembly (Robinson and Schultz, 2003; Tagami et al., 2004).

Genetic analysis has demonstrated an essential role for Asf1 in normal cell cycle progression, with mutants accumulating with a $G_2$/M DNA content (Tyler et al., 1999). The fission yeast homolog of Asf1, CIA1, is essential for viability (Umehara et al., 2002). Although *S. cerevisiae ASF1* null mutants are viable, the cells grow slowly and are

sensitive to DNA-damaging and replication-blocking agents (Le et al., 1997; Tyler et al., 1999). This sensitivity may reflect the requirement for Asf1 during chromatin assembly following DNA repair, as the human homolog of Asf1 has been shown to function synergistically with CAF-1 to assemble nucleosomes during nucleotide excision repair in vitro (Mello et al., 2002). Furthermore, the DNA damage checkpoint is activated in yeast *asf1Δ* cells growing under normal conditions (Ramey et al., 2004). The recently identified role for Asf1 during S phase in the presence of DNA damaging agents or replication stress may contribute to this increased sensitivity; Asf1 was found to interact with components of the DNA replication machinery and maintain their association with stalled forks, thereby promoting genome stability (Franco et al., 2005). Collectively, these observations establish roles for Asf1 in replication-dependent, replication-independent, and DNA repair-coupled chromatin assembly (Munakata et al., 2000; Robinson and Schultz, 2003). In addition, Asf1 plays a global role in chromatin disassembly in budding yeast, and in targeted nucleosome disassembly and transcriptional activation of the *PHO5* and *PHO8* phosphate-inducible genes (Adkins and Tyler, 2004; Adkins et al., 2004).

Although most studies of Asf1 have focused on its role in chromatin assembly on a global scale, yeast Asf1 is also required for proper transcriptional activation and repression of the histone genes (Sutton et al., 2001). Histone gene expression takes place exclusively during S phase, with repression during all other stages of the yeast cell cycle requiring the Hir1 and Hir2 proteins (Osley and Lycan, 1987; Sherwood et al., 1993; Spector et al., 1997). Mutation of yeast *ASF1* results in suppression of S phase-specific histone gene activation and failure to repress expression during the rest of the cell cycle (Sutton et al., 2001). This cell cycle regulation is likely mediated through the formation of a proper chromatin structure by a pathway involving both Asf1 and the Hir proteins (Sharp et al., 2001).

The regions of histone chaperones involved in mediating their chromatin assembly, transcriptional, and DNA damage functions are beginning to be elucidated through structural studies. Yeast Asf1 consists of a highly conserved amino (N)-terminal region (Fig. 1.2, A) followed by a long, less conserved poly-acidic tract. The N-terminal 155 amino acid residues of Asf1 are sufficient for all tested in vitro and in vivo functions

performed by Asf1 including chromatin assembly, transcriptional silencing, and response to DNA damage (Daganzo et al., 2003). The crystal structure of this domain reveals that the surface of Asf1 includes a hydrophobic groove flanked on one side by a patch of acidic amino acid residues. This hydrophobic region contains solvent-exposed residues on the highly conserved β7 and β8 strands that represent potential binding sites for histones and other interacting proteins (Fig. 1.2, B). A patch of acidic residues on or in close proximity to the β4 and β5 strands are also hypothesized to contribute to histone binding (aspartate-37, glutamate-39, aspartate-58, and aspartate-77; Fig. 1.2, B; Daganzo et al., 2003). The recently published NMR structure indicates that the core of the Asf1 interface required for this binding involves a patch of highly conserved hydrophobic residues (valine-94 is at its center) that binds to the carboxy (C)-terminal helix of H3 (Mousson et al., 2005). Conserved charged residues in the vicinity also make a contribution to binding (aspartate-54 and asparagine-108). It was found that Asf1 mutations that disrupted H3/H4 binding also led to defects in transcriptional silencing and DNA damage sensitivity (Mousson et al., 2005).

CAF-1 is a histone H3/H4 chaperone that functions synergistically with Asf1 to deposit newly synthesized histones H3/H4 onto DNA in a replication and repair-coupled manner (Stillman, 1986; Smith and Stillman, 1989; Gaillard et al., 1996 Tyler et al., 2001; Mello et al., 2002). It is composed of three highly conserved subunits, Cac1, Cac2 and Msi1 (Cac3). *ASF1* and the *CAC* genes interact genetically, and Asf1 bound to H3/H4 stimulates the chromatin assembly activity of CAF-1 (Tyler et al., 1999; Sharp et al., 2001). CAF-1 and Asf1 are targeted to replication forks and sites of DNA repair through interaction of Cac1 with the DNA polymerase processivity clamp, proliferating cell nuclear antigen (PCNA) (Shibahara and Stillman, 1999; Moggs et al., 2000; Zhang et al., 2000). There is a physical interaction between Asf1 and Cac2 (Tyler et al., 2001; Krawitz et al., 2002; Mello et al., 2002). Deletion of CAF-1 subunits results in an increased sensitivity to UV radiation and a disruption of telomeric silencing (Enomoto and Berman, 1998; Enomoto et al., 1997; Game and Kaufman, 1999; Kaufman et al., 1997; Monson et al., 1997). Deletion of *ASF1* or *CAC* genes also causes increased gross chromosomal rearrangements (GCRs) which indicate a requirement for these CAFs in maintenance of genome stability (Myung et al., 2003; Prado et al., 2004).

In addition to being a CAF, the smallest subunit of CAF-1, Msi1 (Multicopy Suppressor of IRA), is an upstream component of the Ras signal transduction pathway, thus providing a link between chromatin assembly and Ras signaling. Msi1 is a member of a highly conserved family of WD-repeat proteins originally identified in budding yeast as an antagonist of the Ras-cyclic AMP pathway (Ruggieri et al., 1989). Glucose is known to induce the Ras/cAMP pathway of yeast, and the GTP-binding Ras protein plays an important role in the response of cells to nutrient signaling cues. Ras predominantly regulates cAMP-dependent protein kinase (PKA) signaling in yeast, as activation of Ras leads to the production of cAMP, which activates PKA (reviewed in Thevelein and de Winde, 1999). Ira1 inhibits signaling through Ras by promoting the accumulation of inactive Ras-GDP, and growth phenotypes associated with deletion of *IRA1* are suppressed by overexpression of either yeast or human *MSI1* (Ruggieri et al., 1989; Qian et al., 1993). Msi1 also appears to be involved in aspects of histone metabolism independent of chromatin assembly. For example, it has been implicated in both histone acetylation, based on its sequence similarity to a HAT, and in histone deacetylation, based on its association with a HDAC in human cells (Parthun et al., 1996; Verreault et al., 1996). Hennig et al. (2003) also recently demonstrated the requirement for Msi1 during development in Arabidopsis, possibly due to its presence in multiple chromatin modifying complexes. Taken together, the available data indicate that Msi1 plays roles in multiple processes, including chromatin assembly, nutrient signaling, and histone modification.

**ATP-dependent chromatin remodeling factors**

Following histone deposition by chaperones, nucleosomes are spaced by ATP-dependent chromatin remodeling factors (reviewed in Varga-Weisz, 2001; Tsukiyama, 2002; Langst and Becker, 2004). These enzymes use the energy of ATP hydrolysis to alter nucleosome structure. Chromatin remodeling is essential for establishment of normal positioning of nucleosomes. In bulk chromatin this positioning generates a state which is generally repressive for DNA-dependent processes such as transcription (Cairns, 2005). Depending on how they are positioned to start with, the movement of nucleosomes promoted by chromatin remodeling factors may increase or decrease the accessibility of a site for DNA-binding proteins such as transcription factors. Chromatin

remodeling factors often act in concert with sequence specific DNA binding proteins and histone modifying enzymes such as HATs and HDACs. Based on complex composition and function, the well-conserved ATP-dependent chromatin remodeling complexes can be divided into the following classes: the SWI/SNF class, the ISWI class, the INO80 class, the CHD class, and the SWR1 class.

The SWI/SNF class of remodeling factors has been most extensively characterized and includes the SWI/SNF and RSC complexes (Martens and Winston, 2003). The SWI/SNF complex acts directly to both activate and repress transcription through its regulation of chromatin structure (Sudarsanam et al., 2000; Sudarsanam and Winston, 2000; Vignali et al., 2000; Martens and Winston, 2002; Narlikar et al., 2002). Both the SWI/SNF and RSC complexes contain bromodomain subunits, the bromodomain being a motif which binds to acetylated histone tails (Ladurner et al., 2003; Matangkasombut and Buratowski, 2003; Haynes et al., 1992; Jeanmougin et al., 1997). *Drosophila* Asf1 has been detected in a complex which includes subunits of the SWI/SNF complex, Brahma (Moshkin et al., 2002). Genetic interactions between fly *asf1* and the genes encoding several subunits of Brahma have also been documented (Moshkin et al., 2002).

Bdf1 is a double bromodomain-containing subunit of both the SWR1 remodeling complex and the transcription initiation factor, TFIID (Matangkasombut et al., 2000; Krogan et al., 2003b; Kobor et al., 2004; Mizuguchi et al., 2004). The SWR1 complex is involved in transcriptional regulation through histone exchange. Recent work demonstrates that both the SWR1 and INO80 chromatin remodeling complexes are recruited to sites of DNA repair (Downs et al., 2004; Morrison et al., 2004; van Attikum et al., 2004). Recruitment of these complexes requires phosphorylation of histone H2A, a marker of DNA damage sites (Downs et al., 2000).

Biochemical and genetic studies have implicated ISWI group members in numerous nuclear processes. These include regulation of overall chromosome structure, chromatin assembly, DNA replication, and transcriptional activation and repression (Deuring et al., 2000; Fyodorov et al., 2004; Collins et al., 2002; Poot et al., 2004; Badenhorst et al., 2002; Yasui et al., 2002; Corona and Tamkun, 2004). ISWI-family members have a C-terminal SANT domain; this domain has been implicated in binding to

histone tails (Boyer et al., 2002). *S. cerevisiae* has two ISWI family members, Isw1 and Isw2, which form different complexes (Tsukiyama et al., 1999). Isw2, together with its binding partner, Itc1, compose a remodeling complex that interacts efficiently with both naked DNA and nucleosomal arrays in an ATP-dependent manner (Tsukiyama et al., 1999; Gelbart et al., 2001). Isw2 is required for transcriptional repression of early meiotic genes, a function that is dependent on the Ume6 transcription factor (Goldmark et al., 2000). Binding of Ume6 to a specific DNA sequence recruits the Isw2 complex to chromatin to facilitate its transcriptional regulation of target genes. ATP-dependent remodeling by Isw2 in cooperation with histone deacetylation by the Rpd3-Sin3 HDAC complex alters the chromatin structure to establish transcriptional repression of early meiotic genes during mitotic growth (Goldmark et al., 2000). In addition, microarray and other transcription studies have shown that Isw2 is required for repression of *INO1*, a-cell specific genes, and numerous other genes involved in cellular metabolism and the stress response (Kent et al., 2001; Ruiz et al., 2003; Fazzio et al., 2001). The chromatin state of target genes that are remodeled by Isw2 is relatively stable, despite the transient association of Isw2 with these loci (Gelbart et al., 2005). Although ISWI is essential in higher organisms (Deuring et al., 2000; Badenhorst et al., 2002; Stopka and Skoultchi, 2003), deletion of Isw1 or Isw2 separately or in combination is not lethal in budding yeast (Tsukiyama et al., 1999).

Another method by which chromatin structure is altered is through incorporation of histone variants (Krude, 1995; Kamakaka and Biggins, 2005; Sarma and Reinberg, 2005). This incorporation plays a key role in regulating chromosome regions and cellular memory of transcriptional states. In contrast to expression of the core histones which is restricted to S phase, variants of H2A and H3 are synthesized throughout the cell cycle. Amino acid variations in the histone variants are located in the histone fold domain (HFD) involved in histone-histone and histone-DNA interactions, or at the N-terminus or C-terminus of the protein (Fig. 1.3). For example, H2A.Z differs from canonical H2A in several residues near the C-terminus. Like their core histone counterparts, histone variants are also subject to post-translational modifications including acetylation, phosphorylation, and methylation, in order to further alter chromatin dynamics (reviewed by Kamakaka and Biggins, 2005).

Histone variants are incorporated into chromatin in a replication-coupled or transcription-coupled manner, or by histone exchange (Fig. 1.4). Histone exchange serves to remove epigenetic marks on histones, such as the relatively stable methylation mark, in order to 'reset' the transcriptional state of a gene. The exchange of histones also facilitates the incorporation of variant histones that play a specialized role in gene regulation. The SWR1 complex is required for recruitment and exchange of H2A.Z into both transcribed and non-transcribed regions (Krogan et al., 2003b, Mizuguchi et al., 2004; Kobor et al., 2004). The H2A.Z histone variant in budding yeast is encoded by the *HTZ1* gene. Htz1 acts in concert with other SWR1 complex components to elicit changes in chromatin structure that result in transcriptional activation or repression (Meneghini et al., 2003; Krogan et al., 2003b; Kobor et al., 2004; Mizuguchi et al., 2004). Two key SWR1 complex components that copurify and functionally interact with Htz1 are the SWI/SNF-related ATPase, Swr1, and TFIID bromodomain-containing subunit, Bdf1 (Krogan et al., 2003b; Mizuguchi et al., 2004; Kobor et al., 2004).

Htz1 is involved in the formation of boundaries that, for example, inhibit spreading of transcriptionally silenced chromatin from telomeres in yeast (Meneghini et al., 2003). Evidence for gene-specific transcriptional regulation by Htz1 comes from experiments showing that it is present at genes involved in phosphate and galactose metabolism (Adam et al., 2001; Santisteban et al., 2000; Krogan et al., 2003b). Interestingly, Htz1 crosslinks to both the promoter and the 3' polyadenylation sites of these target genes. Htz1 localizes to the promoters of some genes during repressive conditions and is then lost upon induction (Leach et al., 2000; Santisteban et al., 2000; Krogan et al., 2003b; Larochelle and Gaudreau, 2003). Based on the requirement for Htz1 for recruitment of the transcription machinery and induction of these genes, it has been speculated that the presence of Htz1 enables rapid activation of target genes (Santisteban et al., 2000; Larochelle and Gaudreau, 2003; Kamakaka and Biggins, 2005). However, little is known about other gene targets of Htz1 and how its presence regulates transcriptional kinetics (repression and/or activation) at these genes.

Another histone variant, H3.3, is deposited into chromatin in a replication-independent manner by a complex containing Asf1 and the Hir protein complex, HIRA, in higher organisms (Ahmad and Henikoff, 2002; Tagami et al., 2004). The H3.3 variant

is enriched at transcriptionally active chromosomal regions which include the promoters of active genes in mammalian cells (Ahmad and Henikoff, 2002; Chow et al., 2005). Together with H3 acetylation and H3 lysine-4 methylation, H3.3 deposition forms a stable epigenetic mark that persists during mitosis (Chow et al., 2005). This further underscores the central role that chromatin assembly and histone modifications play in regulating transcriptional states and their inheritance. Interestingly, although only one H3 histone is expressed in budding yeast, it is most similar to the vertebrate H3.3 variant (Baxevanis and Landsman, 1998) which can be deposited into chromatin both during and outside of S phase (Ahmad and Henikoff, 2002).

**Transcription is regulated by chromatin**

Although individual nucleosomes are refractory to transcription, the packaging of DNA into chromatin is not inherently repressive in nature. Nucleosome structure is dynamic and can be altered by ATP-dependent remodeling factors and histone-modifying enzymes such as HATs and HDACs (reviewed in Ehrenhofer-Murray, 2004). The specific histone modification pattern proximal to a given gene is established by histone-modifying enzymes and plays a central role in regulation of gene expression, possibly through formation of a histone code that is read by other proteins to bring about downstream events (Turner, 2000; Jenuwein and Allis, 2001; Fischle et al., 2003). The local configuration of chromatin changes in the course of the activation and repression of a gene, and it has become increasingly evident that living cells possess many factors involved in alteration of nucleosome structure in order to regulate transcription. Following deposition of histones by chaperones, remodeling factors such as ISWI and SWI/SNF catalyze alterations in nucleosome positioning that alter the ability of histone-modifying enzymes and transcription factors to bind to the DNA (Fig. 1.5). The ordered combination of these activities may result in structural alterations that allow a repressed promoter to become transcriptionally active.

The organization of nucleosomes can inhibit several processes that occur during gene regulation. Nucleosomes inhibit transcription at two stages: transcription initiation and promoter-proximal transcriptional elongation (Orphanides et al., 1998). Formation of the preinitiation complex (PIC) is most commonly initiated by specific binding of transcription initiation factor IID (TFIID) to the promoter TATA box, and is a crucial

step in transcriptional regulation (Parker and Topol, 1984; Horikoshi et al., 1988a, b). TFIID is a multisubunit complex comprised of the TATA box binding protein, TBP, and several TBP-associated factors ($TAF_{II}$s) (Dynlacht et al., 1991; Takada et al., 1992; Sanders and Weil, 2000). In yeast, the largest subunit of TFIID is $TAF_{II}145$, which functions in association with Bdf1, a protein that binds with high affinity to N-terminally acetylated histone tails (Matangkasombut et al., 2000; Jacobson et al., 2000; Ladurner et al., 2003; Matangkasombut and Buratowski, 2003). $TAF_{II}$s are not strictly required for TBP association and PIC formation at all promoters (Kuras et al., 2000; Li et al., 2000). Furthermore, the PIC can also be formed at promoters that do not contain a consensus TATA box, for example through binding of $TAF_{II}145$ to a specific DNA sequence (Butler and Kadonaga, 2002). TFIID access to the promoter is regulated by the physical barrier created by nucleosomes. In addition, the chromatin modification profile at a promoter plays a central role in regulating PIC formation.

Subsequent to PIC formation, TFIID recruits the RNA polymerase II ($RNAP_{II}$) holoenzyme, containing initiation factors and the Srb-Mediator complex that functions as a transcriptional coactivator (Hampsey and Reinberg, 1999). An alternative form of $RNAP_{II}$, the Paf1 complex, is associated with both transcription initiation and elongation, indicating that it may play roles at multiple stages of transcription (Shi et al., 1997; Pokholok et al., 2002; Squazzo et al., 2002; Krogan et al., 2003a). Both negative and positive factors control elongation events, in addition to proteins that modify chromatin structure in order to regulate elongation (Conaway et al., 2000; Winston, 2001). These enzymes may work together, as evidenced by the genetic interaction of the gene encoding histone chaperone Asf1 with genes involved in transcriptional elongation. *DST1*, *PAF1* and *CDC73* encode proteins which function in elongation (Hartzog, 2003). Double mutants of each of these three genes with *asf1Δ* have more severe phenotypes than the respective single mutants (Formosa et al., 2002; Krogan et al., 2003b); this is consistent with the possibility of a functional interaction between Asf1 and these elongation factors. Recent evidence demonstrates that nucleosome depletion occurs upon transcriptional activation of target genes such as *PHO5* (Reinke and Horz, 2003; Boeger et al., 2004; Lee et al., 2004). Asf1 expression is required for nucleosome disassembly at the *PHO5*

promoter, thereby further implicating this histone chaperone in transcriptional regulation (Adkins et al., 2004).

**Transcriptional regulation by Asf1**

Active transcription, which is generally inhibited by incorporation of promoter DNA into chromatin, may directly involve Asf1. Human Asf1 physically interacts with the TAF1 ($TAF_{II}250$) subunit of transcription initiation factor TFIID, and in budding yeast Asf1 exists in a complex with TFIID and can directly bind to its bromodomain-containing Bdf1 and Bdf2 subunits (Chimura et al., 2002). *ASF1* and *BDF1* of yeast also interact genetically (Chimura et al., 2002). Coupled with recent reports that HAT-containing complexes and nucleosome remodeling factors affect binding of TFIID to nucleosomal templates (Agalioti et al., 2000; Lomvardas and Thanos, 2001), this suggests a role for Asf1 in the regulation of interactions between TFIID and nucleosomes during transcription initiation. Furthermore, HATs and remodeling factors also contain bromodomains (Jeanmougin et al., 1997; Haynes et al., 1992); therefore Asf1 may regulate transcription through interactions with these domains in chromatin-modifying complexes as well. Collectively these observations raise the possibility that Asf1 directly participates in targeted chromatin metabolism by mechanisms that are independent of its global function in chromatin assembly. For example, there may be a pool of Asf1 molecules that interact with CAF-1 and perform chromatin assembly. A separate pool of Asf1 may interact with chromatin remodelers and components of the transcriptional machinery in order to regulate the expression of specific genes.

Chromatin plays an important role in a form of transcriptional regulation called silencing, in which complete repression of transcription occurs by mechanisms that involve position-dependent, gene-independent changes in chromatin structure over large regions of the genome (reviewed in Moazed, 2001; Rusche et al., 2003). In *S. cerevisiae*, silencing occurs at the mating-type loci (*HML* and *HMR*), the rDNA array, and near the telomeres (Rusche et al., 2003). During M phase the chromatin structure of telomeres is stabilized (silenced) such that genes placed in their vicinity cannot be transcribed when the cell enters $G_1$. Sir proteins are important structural components of the chromatin at silenced regions. In higher organisms the genome is organized into cytologically defined regions of euchromatin and heterochromatin. Euchromatin is chromatin that is

decondensed and is thought to be transcriptionally active. Active genes in this region contain histones that are hyperacetylated and enriched for methylation on H3 lysine (K)-4, K-36, and K-79. Heterochromatin is highly compacted chromatin with regions of silenced genes that contain hypoacetylated histones.

Transcriptional silencing in yeast is mediated by a number of proteins in addition to the Sirs, including those that modify the histone N-termini and those involved in histone deposition onto DNA. Yeast *asf1Δ* mutants display minor defects in telomeric gene silencing, a reflection of a requirement for Asf1 in the assembly of silenced chromatin (Le et al., 1997; Singer et al., 1998; Tyler et al., 1999; Sharp et al., 2001). The Something About Silencing (SAS) complex is a histone acetyltransferase (HAT) complex involved in transcriptional silencing in *S. cerevisiae* (Reifsnyder et al., 1996; Ehrenhofer-Murray et al., 1997; Xu et al., 1999; Meijsing and Ehrenhofer-Murray, 2001). The SAS complex blocks the spread of silencing complexes through acetylation of K-16 on histone H4, thereby antagonizing the spread of key promoters of transcriptional silencing which include the Sir proteins (Sutton et al., 2003; Shia et al., 2005). Asf1 and CAF-1 physically, genetically, and functionally interact with SAS complex components (Meijsing and Ehrenhofer-Murray, 2001; Osada et al., 2001). However, the possibility that SAS-dependent HAT activity has a role in the chromatin assembly functions performed by Asf1 and CAF-1 has yet to be fully explored.

Further evidence supports a role for Asf1 and CAF-1 in transcriptional silencing through an interaction with the Hir proteins. The Hir proteins bind histones and have been implicated in replication-independent nucleosome assembly (Lorain et al., 1998; Ray-Gallet et al., 2002; Tagami et al., 2004). Inaddition, the Hir proteins are hypothesized to contribute to transcriptional silencing through their regulation of histone gene expression (Kaufman et al., 1998). They have silencing functions that partially overlap with CAF-1, and Hir1 and Hir2 physically and genetically interact with Asf1 (Kaufman et al., 1998; Qian et al., 1998; Sharp et al., 2001; Sutton et al., 2001).

**The DNA damage response**

In order to preserve genomic integrity, checkpoint responses arrest the cell cycle in response to DNA damage and replication blocks until the damage is repaired (reviewed in Zhou and Elledge, 2000). This ensures accurate transmission of genetic

information to progeny and overall survival of the organism. The cell cycle is comprised of four stages: 1) the $G_1$ (Gap 1) phase of cell growth, 2) the S phase of DNA replication, 3) the $G_2$ (Gap 2) phase of growth, and 4) the M phase of mitosis and cell division (Fig. 1.6). The DNA damage checkpoints can be invoked at three stages of the cell cycle, including the $G_1$/S transition, during S phase, and at the $G_2$/M boundary. DNA damage checkpoints are essential for allowing time for DNA repair to be performed, as well as for the activation of machineries that carry out this process (Rouse and Jackson, 2002). In addition to cell cycle arrest, the response to DNA damage involves post-translational modifications of proteins involved in this signaling cascade, transcriptional induction of genes required for DNA repair, and changes in chromatin structure at the site of damage.

The alkylating agent, methyl methanesulfonate (MMS), is an electrophile that adds methyl groups to nucleic acid bases to create adducts and apurinic sites (reviewed in Bilsland and Downs, 2005). These become single and double strand breaks (DSBs) during processing of the lesions (Schwartz, 1989). MMS causes single stranded breaks at low concentrations, but double strand breaks (DSBs) at higher concentrations due to the proximity of the single strand breaks. DSBs that are not properly and quickly repaired can be deleterious to the integrity of the genome by leading to gene rearrangements, deletions, or aneuploidy. One commonly used method of creating DNA damage specifically during S phase is treatment of cells with the replication inhibitor, hydroxyurea (HU). HU directly inhibits the formation of dNTPs, leading to collapse of the replication fork and generation of DSBs. In fact, although the pathways that respond to DNA damage induced by MMS and HU act in parallel, there is also cross-talk between them. For example, MMS-induced DNA alkylation can cause stalling of replication forks by mimicking the effects of dNTP depletion and causing stalling of the DNA polymerase complex upon confrontation of an adduct. Furthermore, replication forks arrested as a result of HU treatment may result in strand breakage.

DNA damage response signal transduction pathways are composed of highly conserved sensors, transducers, and effectors. Following detection of DNA damage, protein kinases such as ATM (Tel1) and ATR (Mec1) elicit the DNA damage response by initiating a cascade of protein phosphorylation. Activation of the Mec1 protein kinase leads to the phosphorylation and activation of another essential checkpoint kinase,

Rad53, that plays multiple roles in the response to genotoxic stress. The Mec1/Rad53 pathway is required for cell cycle arrest, activation of downstream effector response proteins, and transcriptional induction of genes that increase the capacity of the cell for repair (Weinert et al., 1994; Sanchez et al., 1996; Sun et al., 1996, 1998; Desany et al., 1998; Emili, 1998; Santocanale and Diffley, 1998; Shirahige et al., 1998; Huang et al., 1998; Schwartz et al., 2003). These latter genes include the *RNR* genes which encode subunits of Ribonucleotide reductase (Rnr), the enzyme that catalyzes the rate-limiting step in deoxyribonucleotide (dNTP) and DNA synthesis (reviewed by Reichard, 1988). In S. *cerevisiae*, Rnr is composed of four subunits, encoded by *RNR1*, *RNR2*, *RNR3* and *RNR4* (Elledge et al., 1992, 1993; Huang and Elledge, 1997).

Transcriptional regulation of the Rnr subunit encoded by *RNR3* has been well-characterized and involves numerous cellular factors and chromatin-dependent steps. *RNR3* transcription is governed by specific binding of the Crt1 repressor to X box DNA binding sequences in the promoter, which then recruits the Ssn6-Tup1 corepressor complex (Huang et al., 1998). These corepressor proteins are required for precise *RNR3* nucleosome positioning and inhibition of transcription (Li and Reese, 2001). Nucleosome positioning across the promoter and coding region of *RNR3* is further mediated by the Isw2 chromatin remodeler, a factor which crosslinks to *RNR3* independent of the Crt1-Ssn6-Tup1 complex (Zhang and Reese, 2004a). Repression also requires the Hda1 histone deacetylase and components of the Mediator sub-complex of $RNAP_{II}$ (Zhang and Reese, 2004b).

Activation of the DNA damage checkpoint results in phosphorylation of the Crt1 repressor, which in turn causes the Crt1-Ssn6-Tup1 complex to be released from the *RNR3* promoter; this event alleviates transcriptional repression (Huang et al., 1998). Chromatin structure alteration and transcriptional activation of *RNR3* further involves the action of the SWI/SNF chromatin remodeling complex as mediated by $TAF_{II}$s and the general transcription machinery (Sharma et al., 2003). Roles for the cell cycle-regulatory transcription factor, SCB-binding factor (SBF), and the protein kinase, Hrr25 have also been demonstrated during DNA damage-induced expression of *RNR3* (Ho et al., 1997). A model showing the factors involved in *RNR3* chromatin structure regulation and transcriptional repression and activation is presented in Figure 1.7.

In addition to transcriptional regulation, Rnr activity is regulated by binding of the Sml1 repressor to the Rnr enzyme (Zhao et al., 1998, 2000; Chabes et al., 1999). During the response to DNA damage Sml1 is phosphorylated and removed in a Mec1/Rad53-dependent manner, thereby facilitating derepression of Rnr activity (Zhao et al., 2001). Finally, Rnr is also allosterically regulated by dATP feedback inhibition (Reichard et al., 2000). The existence of multiple layers of control of Rnr activity underscores the importance of dNTP regulation in a number of key processes, including replication, recombination and repair. Indeed, proper regulation of Rnr is essential for the maintenance of genome stability, as an elevated dNTP pool has been shown to be mutagenic in *S. cerevisiae* (Chabes et al., 2003).

Asf1 is thought to function as a key component in the response to DNA damage by virtue of a physical and functional interaction with the checkpoint kinase, Rad53. Asf1 and Rad53 exist in a dynamic complex that dissociates in response to replication blocks and DNA damage (Emili et al., 2001; Hu et al., 2001). This complex does not include histones H3 and H4 (Emili et al., 2001) and it is not capable of supporting in vitro histone deposition. When cells experience genotoxic stress, Asf1 is released from the Rad53 complex and becomes competent for nucleosome assembly coupled to DNA repair (Emili et al., 2001; Hu et al., 2001).

We have identified a direct role for Asf1 in transcriptional regulation of the DNA damage response gene, *RNR3*, and identified chromatin factors involved in the metabolism of *RNR3*-associated Asf1. Furthermore, the function of Asf1 at *RNR3* is subject to physiological regulation by DNA damage signals. These findings further our understanding of CAF function and set the stage for a re-evaluation of the role of histone chaperones in gene regulation.

**Part II: Physiological regulation of histone modification**

**Histone modification and transcriptional regulation**

One well-characterized mechanism of chromatin structure alteration involves post-translational histone modification. Core histones have flexible N-terminal tails that extend outward from the nucleosome and are subject to a number of post-translational modifications, including acetylation, methylation, and ubiquitination of lysine residues, phosphorylation of serine and threonine residues, and methylation of arginine residues (reviewed in Fischle et al., 2003). The complex pattern of histone modifications serves as a signaling platform for nuclear processes through regulation of chromatin structure and function (Berger, 2002; Iizuka and Smith, 2003). Histone acetylation is the best characterized histone modification. Acetylation of lysine residues is catalyzed by histone acetyltransferases (HATs), which act in opposition to histone deacetylases (HDACs). These enzymes may be components of large multi-protein complexes that possess other chromatin modifying subunits, or they may act alone (Hassan et al., 2001). On a global scale, HAT and HDAC-catalyzed reactions occur continuously, resulting in an equilibrium of bulk histone acetylation. It is thought that acetylation neutralizes the positive charges on the lysine epsilon amino groups on the histone tails, thus loosening histone-DNA interactions and opening the chromatin fiber to permit binding and function of the transcriptional apparatus (Workman and Kingston, 1998).

Chromatin-modifying enzymes can be recruited to genes by DNA sequence-specific transcription factors to regulate transcriptional activity. The specific pattern of histone modifications that is established, known as a 'histone code' is also involved in recruitment of proteins (Jenuwein and Allis, 2001; Fischle et al., 2003). For example, proteins containing one or more bromodomains specifically recognize acetylated histones (Ladurner et al., 2003; Matangkasombut and Buratowski, 2003). Therefore, histone modifications such as acetylation regulate both overall chromatin structure and recruitment of specific factors. In addition to gene-localized effects, histone modifications also control the establishment and maintenance of larger, specialized regions of the genome such as heterochromatin (Grewal and Elgin, 2002). Changes in the modification state of chromatin may facilitate incorporation of histone variants or their eviction. Chromatin modifications often create novel binding interfaces for

regulators of transcription and DNA repair, as well as other chromatin-modifying proteins (reviewed by Khorasanizadeh, 2004).

Distinct post-translational histone modifications can function synergistically or antagonistically to regulate transcription, and are correlated with the execution of a variety of cellular events. Examples include the involvement of phosphorylated H3 serine-10 in transcription activation, mitosis, and chromosome condensation and decondensation (Nowak and Corces, 2004; Prigent and Dimitrov, 2003). In cycling cells, H3-S10 phosphorylation at specific promoters is often associated with gene activation (Lo et al., 2001), whereas during mitosis this mark correlates with chromosome condensation (Hendzel et al., 1997; Van Hooser et al., 1998). H3-S10 is phosphorylated during mitosis and meiosis where it is thought to promote proper chromosome condensation and segregation (Hans and Dimitrov, 2001). Furthermore, phosphorylation of H3-S10 has also been mechanistically linked to H3 acetylation (Lo et al., 2000).

As noted previously, enzymes that modify chromatin can be recruited by the transcription machinery. For example, complexes containing the HAT, Gcn5, are recruited by transcriptional activators (Grant et al., 1999; Brown et al., 2001; Martinez et al., 2001), and the Set1 methyltransferase is recruited to active genes by the $RNAP_{II}$-associated Paf1 complex (Ng et al., 2003; Krogan et al., 2003a). McKittrick et al. (2004) proposed that nucleosome assembly complexes may also recruit histone-modifying enzymes to sites of chromatin assembly. Evidence in support of this idea comes from studies showing that the SAS H4 acetyltransferase complex associates with Asf1 (Meijsing and Ehrenhofer-Murray, 2001; Osada et al., 2001).

It is possible to manipulate the extent of tail modification of the core histones on a global scale by artificial means. In budding yeast for example deletion of HAT genes results in global deacetylation of target histones, and deletion of HDACs the reverse (Carmen et al., 1999; Clarke et al., 1999; Howe et al., 2001; Reid et al., 2000; Rundlett et al., 1996; Vogelauer et al., 2000). The effects of HDAC deletion on global histone acetylation in yeast can be mimicked in mammalian cells by treatment with HDAC inhibitors such as butyrate, trichostatin A, or trapoxin (Yoshida et al., 1990). Furthermore, mutations that partially cripple an H3-S10 kinase (Ipl1p) or the Glc7p catalytic subunit of an H3-directed type 1 protein phosphatase (PP1) globally affect the

accumulation of S10-phosphorylated H3 in yeast (Hsu et al., 2000). Consistent with the general observation that the global state of histone modification is sensitive to artificial manipulation of histone-modifying enzymes, it has been found that core histone acetylation and phosphorylation are physiologically regulated on a global scale.

**Stationary phase**

$G_1$ haploid yeast cells begin the mitotic cell cycle with one copy of each chromosome (1n) which is then doubled (2n) during DNA replication and then restored to 1n following cytokinesis in M phase. The internal and external cellular conditions are surveyed during $G_1$ phase before a cell commits to a new round of DNA replication, mitosis, and cell division. This commitment occurs at a point known as 'Start' in budding yeast, and the 'Restriction point' in mammals (Fig. 1.6). When nutrients are available and other conditions are permitting, the cell will pass Start and proceed through the stages of the cell cycle. Nutrient deprivation prevents passage through Start and triggers yeast cells to exit the $G_1$ phase of the cell cycle into stationary phase ($G_0$; Fig. 1.6). Only after nutrients again become available will yeast cells re-enter the mitotic cell cycle from $G_0$ (Gray et al., 2004).

Cell cycle exit into stationary phase occurs through a reprogramming of regulatory networks and remodeling of intracellular structures and processes analogous to differentiation in higher eukaryotes (Herman, 2002; Gray et al., 2004). There is a cessation of growth in which cells arrest in a non-proliferative state with a 1n DNA content. In budding yeast this involves a switch in energy metabolism from fermentation to mitochondrial respiration. Depletion of glucose triggers an extensive change in metabolism in which global transcription and translation are downregulated (Boucherie, 1985; Choder, 1991; Jona et al., 2000). For example, the overall transcription rate is three to five times lower than in exponentially growing cultures (Choder, 1991). A complex transcriptional reprogramming characterized by repression as well as induction of specific target genes required for survival during stationary phase occurs (Werner-Washburne et al., 1993, 1996; DeRisi et al., 1997). Chromosomes in $G_0$ cells adopt a folded conformation that causes them to sediment more slowly than chromosomes isolated from exponentially growing cells (Piñon and Salts, 1977; Piñon, 1978; Werner-Washb[illegible]e et al., 1993, 1996). The biochemical, morphological, and physiological

changes associated with stationary phase appear to promote cellular survival for prolonged periods of time (Werner-Washburne et al., 1993). Yeast is a good system for studying the quiescent state of eukaryotic cells as it is inducible by nutrient limitation and many genes required for survival during and exit from stationary phase have human homologues (Martinez et al., 2004). Like yeast cells, mammalian cells have the ability to respond to starvation by entering into a quiescent state (Longo and Fabrizio, 2002).

**The Anaphase Promoting Complex**

Eukaryotic cells possess a highly specific system, known as the ubiquitin proteasome pathway, for the selective degradation of proteins. Central to this pathway is the covalent attachment of lysine-48-linked polyubiquitin chains onto substrate proteins, which targets them for degradation by the 26 S proteasome (Hochstrasser, 1996; Hershko, 1997). Ubiquitin-mediated proteolysis is required for a variety of cellular processes. Ubiquitination of proteins is a multistep process involving three reactions, as three classes of activities cooperate in catalyzing protein ubiquitination (Fig. 1.8). The E1 enzyme is the ubiquitin activating enzyme: Ubiquitin is activated in an ATP-dependent manner through formation of a high energy thioester bond between its C-terminal glycine residue and the active site cysteine residue in the E1. It is the E2 ubiquitin conjugating enzyme to which ubiquitin is then transferred by a process in which ubiquitin forms a new thioester bond with the active site of the E2. Next, the E3 ubiquitin protein ligase catalyzes formation of a stable isopeptide linkage between ubiquitin's C-terminus and one or more lysine residues of the substrate. Multiple rounds of ubiquitin transfer to lysine residues within ubiquitin itself leads to the formation of a polyubiquitin chain recognized by the 26S proteasome, resulting in substrate proteolysis. The 26 S proteasome is a multisubunit protease specific for multiubiquitinated substrates (Coux et al., 1996; Baumeister et al., 1998). Ubiquitination, rather than degradation, is cell cycle regulated as the 26S proteasome is active throughout the cell cycle (Mahaffey et al., 1993).

The important roles performed by the E3 enzyme include determination of the substrate specificity of ubiquitination and recruitment of the appropriate E2, as the E3 enzyme plays a crucial role in substrate selectivity and timing of degradation. The anaphase promoting complex (APC) is a highly conserved multisubunit protein ubiquitin

ligase (E3) that targets key cell cycle regulators for degradation by the proteasome (reviewed in Passmore, 2004; Castro et al., 2005). The APC appears to have a similar composition in all eukaryotes (Peters et al., 1996; Yu et al., 1998; Zachariae et al., 1998; Grossberger et al., 1999). The APC is active during the M and $G_1$ phases of the cell cycle (Fig. 1.6), and is required for the anaphase to metaphase transition and exit from mitosis (Zachariae and Nasmyth, 1999; Harper et al., 2002; Peters, 2002). Principal targets of the APC in yeast regulate progression from M phase to $G_1$, as well as events during $G_1$. The APC is also active in quiescent mammalian cells (Gieffers et al., 1999). The activity of the APC is tightly cell cycle regulated, and precise regulation is crucial to faithful cell cycle progression. The availability of nutrients influences coordination of cell division and cell growth, and nutritional conditions have been shown to influence APC function (Irniger et al., 2000).

The APC is composed of 13 stably associated subunits in budding yeast, and 11 in humans (Table 1.1). Many subunits possess well-conserved motifs that serve specialized functions. As an E3 enzyme, the APC is responsible for transferring ubiquitin from an E2 (Ubc4 in *S. cerevisiae*) to its substrate. Many E3 ubiquitin ligases have a RING-H2 finger domain in their catalytic core (Jackson et al., 2000; Pickart, 2001). In the APC, the Apc2 and Apc11 subunits comprise the catalytic core, with Apc11 catalyzing the recruitment of the E2 enzyme to the APC (Gmachl et al., 2000; Leverson et al., 2000). Apc2 posses a cullin domain, and Apc11 possesses a RING-H2 finger domain. Both domains are found in subunits of several ubiquitination complexes, and Cullin domains have been shown to bind to RING-H2 finger motifs (Weissman, 2001). The Doc domain in the Apc10 (Doc1) subunit is found in several other proteins involved in ubiquitination that also possess domains such as cullin, RING-H2 finger, and HECT domains (Hwang and Murray, 1997; Kominami et al., 1998; Grossberger et al., 1999). Tetratricopeptide repeat (TPR) motifs found in three APC subunits (Cdc16, Cdc23, and Cdc27) appear to act as protein-protein interaction domains and serve a scaffold function (Lamb et al., 1995; Blatch and Lassle, 1999).

The Apc10 subunit was originally identified in *S. cerevisiae* in a genetic screen for mutants that were defective in degradation of the Clb2 mitotic cyclin (Hwang and Murray, 1997). Apc10 is highly conserved in many organisms (Fig. 1.9; Kominami et

al., 1998). Apc10 appears to be essential for APC function in mammals (Grossberger et al., 1999; Wendt et al., 2001), and budding yeast cells lacking Apc10 have impaired E3 ligase function (Passmore et al., 2003). Apc10 is associated with the APC throughout the cell cycle and directly associates with the Apc11 catalytic subunit (Grossberger et al., 1999; Tang et al., 2001). The C-terminus of human Apc10 binds to the TPR-containing Cdc27 subunit (Wendt et al., 2001). Apc10 appears to function as a regulatory component as it is required for interaction of substrates with an APC-coactivator complex. Activator proteins associate with the APC in a cell cycle-dependent manner and are thought to interact directly with target proteins (Peters, 2002; Harper et al., 2002). Its role in substrate recognition suggests that Apc10 may play a role similar to the activator proteins in regulation of binding of specific substrates. Despite its requirement for substrate recognition, Apc10 is not necessary for the association of the activator with the APC (Passmore et al., 2003). Based on its direct interaction with Apc11, Apc10 may be in a position to hold substrates in close proximity to the catalytic core of the APC (Tang et al., 2001). *APC10* deletion inactivates APC function without disrupting complex formation (Passmore et al., 2003; Grossberger et al., 1999; Carroll and Morgan, 2002), and therefore it has been postulated that Apc10 is involved in binding to ubiquitin or inducing a conformational change (Passmore, 2004).

Apc10 has been described as a processivity factor based on its ability to limit substrate dissociation (Carroll and Morgan, 2002; Passmore et al., 2003). Apc10 crystal structure determination indicates the possibility of a conserved ligand-binding region that is part of the Doc domain (Au et al., 2002; Wendt et al., 2001). The central core of Apc10 consists of a β-sandwich, an N-terminal loop region, and a C-terminal loop region (Fig. 1.10). The Doc domain is located within the β-sandwich jelly roll region, a structural motif that is believed, in other proteins, to mediate biomolecular interactions (Au et al., 2002; Wendt et al., 2001).

Apart from its core components, the APC also transiently associates with the activator proteins, Cdc20 and Cdh1, in a cell cycle-dependent manner. Their sub-stoichiometric association with the APC controls its activity and substrate specificity (Peters, 2002; Harper et al., 2002). Cdc20 associates with the APC and activates it during mitosis prior to anaphase, whereas Cdh1 binding maintains the APC in an active

state throughout late mitosis and $G_1$. Another activator, Ama1, is required for APC function during meiosis (Cooper et al., 2000). The Cdc20 and Cdh1 activators determine the timing of activity and substrate specificity of the APC, and bind directly to target substrates (Schwab et al., 2001; Burton and Solomon, 2001; Pfleger et al., 2001; Hilioti et al., 2001). In addition to APC regulation by association with specific activators, APC activity is also regulated by subunit phosphorylation and the spindle assembly checkpoint (reviewed by Castro et al., 2005). Targets of the APC include the Pds1 securin protein, whose destruction is required for sister chromatin separation at anaphase, and mitotic cyclins such as Clb2, which must be degraded before mitotic exit (Cohen-Fix et al., 1996; Ciosk et al., 1998; Irniger, 2002). APC substrates during the $G_1$ phase of the cell cycle include its activator, Cdc20, as well as Aurora A kinase (Pfleger and Kirschner, 2000; Littlepage and Ruderman 2002, Castro et al., 2002a, b).

All APC substrates identified to date possess conserved sequence elements called destruction (D) boxes, destruction box activating (A) boxes, or KEN box motifs, singly or in combination (Glotzer et al., 1991; Pfleger and Kirschner, 2000; Castro et al., 2002b; Littlepage and Ruderman, 2002). The D box and KEN box sequences are critical to the interaction between APC substrates and the Cdc20/Cdh1 coactivator proteins (King et al., 1996; Pfleger and Kirschner, 2000; Hilioti et al., 2001; Burton and Solomon, 2001). The degenerate core D box motif is RxxLxxxxN, although there is some flexibility at the last position (Glotzer et al., 1991; King et al., 1996). However, other elements such as the A box (QRVL) motif located near the D box appear to be required for its proper recognition, as the D box recognition motif may not by itself contain sufficient information to confer ubiquitination of a substrate. The KEN box degradation motif (KENxxxN) has also been identified in a relatively small number of APC substrates (Pfleger and Kirschner, 2000).

The APC has also been found to be required for the degradation of transcription factors. For example, it is involved in TGFβ signaling through degradation of the SnoN transcription factor (Stroschein et al., 2001; Wan et al., 2001). The mammalian SnoN transcription factor is an inhibitor of TGFβ signaling whose expression is regulated by the APC through a mechanism involving an interaction between its Apc10 subunit and Smad3, a key signal transducer of TGFβ that initiates the degradation of SnoN

(Stroschein et al., 2001; Wan et al., 2001; Nourry et al., 2004). SnoN contains a D box which is required for its degradation and interaction with Smad3 (Stroschein et al., 2001). The APC is involved in control of cell proliferation by inducing degradation of the HOXC10 transcription factor (Gabellini et al., 2003). HOXC10 possesses two D box motifs that are required for its timely destruction early in mitosis by the APC (Gabellini et al., 2003). Furthermore, an active APC complex is present in post-mitotic tissues including neurons (Gieffers et al., 1999), and the APC plays a role in establishment of cell polarity during embryonic development in *C. elegans* (Rappleye et al., 2002).

Among the APC targets are the Aurora kinases, a family of mitotic serine/threonine kinases conserved from yeast to humans (Chan and Botstein, 1993; Glover et al., 1995; Schumacher et al., 1998; Roghi et al., 1998; Gopalan et al., 1997; Kimura et al., 1997). They are involved in numerous mitotic processes, including the $G_2$/M transition, chromosome condensation and segregation, and cytokinesis. In budding yeast, the single essential Aurora kinase, Ipl1, localizes to the mitotic spindle and is required for chromosome segregation and cytokinesis (Chan and Botstein, 1993; Biggins et al., 1999; Kim et al., 1999; Shannon and Salmon, 2002; Stern, 2002). Ipl1 also has a role in spindle disassembly (Buvelot et al., 2003). Ipl1 is named for Increased Ploidy. In yeast and animal cells Ipl1/Aurora protein levels fluctuate during the cell cycle with kinetics consistent with degradation by the APC (Gopalan et al., 1997; Kimura et al., 1997; Roghi et al., 1998).

There are three Aurora kinases in mammalian cells, Aurora A, Aurora B, and Aurora C. The activity and expression of the H3-S10 kinase, Aurora A is regulated in a cell cycle-dependent manner, and its timely destruction is controlled by the APC. This enzyme physically interacts with the N-terminal tail of H3 and efficiently phosphorylates S10 in free and nucleosomal H3 (Crosio et al., 2002; Scrittori et al., 2001). During the division cycle, Aurora A is ubiquitinated by the APC and degraded in $G_1$, and is then subsequently re-synthesized (Castro et al., 2002a; Taguchi et al., 2002; Honda et al., 2000; Littlepage and Ruderman, 2002). The destruction of Aurora B during late mitosis has also recently been shown to be mediated by the APC. Aurora B associates directly with the Cdc27 subunit, and its ubiquitination and subsequent degradation is dependent

on its N-terminal KEN and A box motifs (Nguyen et al., 2005). Aurora C function and regulation have not been well-characterized.

A precise balance of competing kinase and phosphatase activities appears to regulate the phosphorylation state of H3-S10 during the cell cycle. It has also been suggested that these enzymes may regulate each other (reviewed by Prigent and Dimitrov, 2003). Protein Phosphatase 1 (PP1) is a highly conserved type-1 serine/threonine phosphatase. The budding yeast PP1 ortholog, Glc7, is essential for viability and plays a role in a number of physiological processes including glycogen metabolism, glucose repression, transcription, translation, mitosis and sporulation (Stark, 1996). In *S. cerevisiae*, the reduced levels of H3-S10 phosphorylation caused by Ipl1 mutation are rescued by mutations in Glc7 (Hsu et al., 2000). Murnion et al. (2001) found that in *Xenopus*, PP1 dephosphorylates not only H3-S10 during interphase, but also Aurora B (Fig.1.11). This dephosphorylation inactivates the ability of Aurora B to act as a kinase. The complex interplay between PP1 and an Aurora kinase, combined with evidence that Aurora degradation is mediated by the APC, suggests the possibility of a regulatory relationship between the APC and PP1.

**Research Overview**

The recent explosion of interest in chromatin-regulated processes has brought such events as chromatin assembly and modification into the scientific limelight. Despite the significant advances made in chromatin research, there is still much to be learned. This thesis describes my efforts to delineate two modes of chromatin regulation, one at a gene-specific level and the other at a global level, in an effort to better understand the complicated mechanisms involved. This work has provided molecular insights into the physiological regulation of two inter-related processes, transcription and the modification state of the histones.

**Chapter 3** presents the results obtained by genome-wide transcriptional profiling of three CAF mutants, and subsequent more detailed analysis of the function of Asf1. We found that histone chaperone Asf1 crosslinks to the *RNR3* DNA damage-inducible gene and regulates its transcription by a chromatin-dependent mechanism. The association of Asf1 with *RNR3* is dynamic and regulated by DNA damage signals.

**Chapter 4** describes the role that other chromatin regulators play in Asf1 metabolism at *RNR3*. These factors include the histone H2A variant Htz1, the transcription initiation factor Bdf1, and the chromatin remodeler Isw2. Each protein is uniquely required for proper regulation of Asf1 association with *RNR3*. Specific genetic, physical, and functional interactions between Asf1 and these regulators were also uncovered.

In **Chapter 5**, I describe our identification of the APC cell cycle regulator as a component of a pathway that sets the global level of histone modification. The APC is required for establishment of proper levels of acetylation of histones H2B, H3 and H4 during cell cycle exit into stationary phase. Global levels of H3-S10 phosphorylation are also controlled by the APC during both the cell cycle and $G_0$ through its regulation of a conserved kinase (Ipl1) and a conserved phosphatase (Glc7).

**Chapter 6** summarizes our studies of gene-specific and global chromatin metabolism in yeast and relates our findings to function in higher organisms. Possible mechanisms of chromatin structure and transcriptional regulation are discussed and future areas of study are suggested. The global role that Asf1 and Apc10 may play in genome

stability and human disease makes them and the processes they regulate potential targets for disease treatment and prevention.

**Table 1.1.** APC subunit nomenclature in *S. cerevisiae* and humans

| *S. cerevisiae* | Humans | Motif |
|---|---|---|
| Apc1 | Apc1/Tsg24 | Rpn1/2 homology |
| Apc2 | Apc2 | Cullin homology |
| Cdc27 | Apc3/Cdc27 | Tetratricopeptide repeats |
| Apc4 | Apc4 | |
| Apc5 | Apc5 | HEAT repeats |
| Cdc16 | Apc6/Cdc16 | Tetratricopeptide repeats |
| | Apc7 | Tetratricopeptide repeats |
| Cdc23 | Apc8/Cdc23 | Tetratricopeptide repeats |
| Apc9 | | |
| Apc10/Doc1 | Apc10 | Doc domain |
| Apc11 | Apc11 | Ring-H2 finger domain |
| Cdc26 | Cdc26 | |
| Apc13/Swm1 | | |
| Mnd2 | | |
| Cdc20[a] | Cdc20 | WD40 repeats |
| Cdh1/Hct1[a] | Cdh1 | WD40 repeats |
| Ama1[b] | | WD40 repeats |

[a] activator proteins that associate with the APC in a cell cycle-dependent manner.
[b] activator protein that associates with the APC during meiosis.

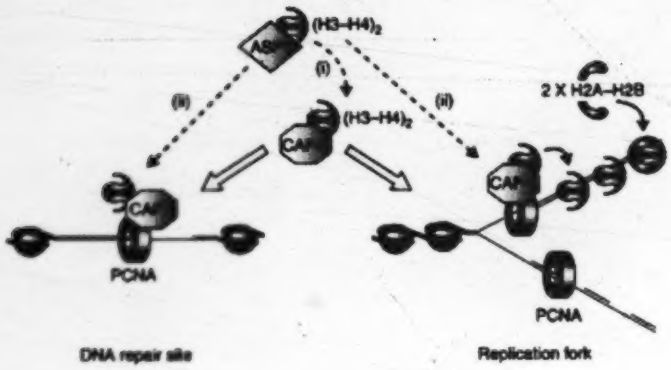


**Figure 1.1. A model for nucleosome assembly coupled to DNA replication and repair.** It has been hypothesized that Asf1 delivers newly synthesized H3 and H4 to DNA directly (ii) or in a CAF-1 dependent manner (i). CAF-1 is targeted to sites of DNA repair and replication through a direct interaction with PCNA. Addition of the H3/H4 tetramer is followed by addition of two H2A-H2B dimers. Figure is from Mello and Almouzni, 2001.

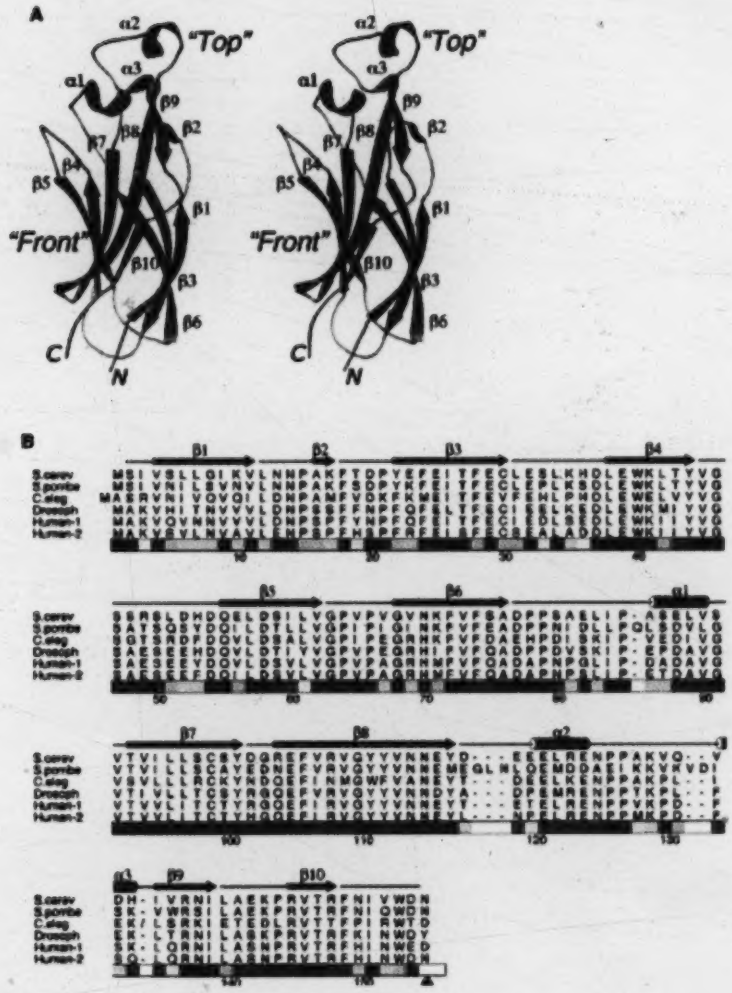


**Figure 1.2. Backbone structure and conservation of the N-terminal 155 amino acids of Asf1. A.** Stereo view of a ribbon diagram of the N-terminal region of Asf1. **B.** Comparison of Asf1 protein sequences and structure. Secondary structure elements match those indicated in panel A. The degree of evolutionary conservation is shown as a gradient of red, orange, yellow and white boxes (red is most highly conserved and white is nonconserved). Most solvent-exposed hydrophobic residues hypothesized to be involved in protein-protein interactions are found on or in close proximity to the β7 and β8 strands (V92, L96, Y112; V45 is found on the β4 strand). Acidic residues that may contribute to histone binding are located on or near the β4 and β5 strands (D37, E39, D58, D77). Figure is adapted from Daganzo et al., 2003.

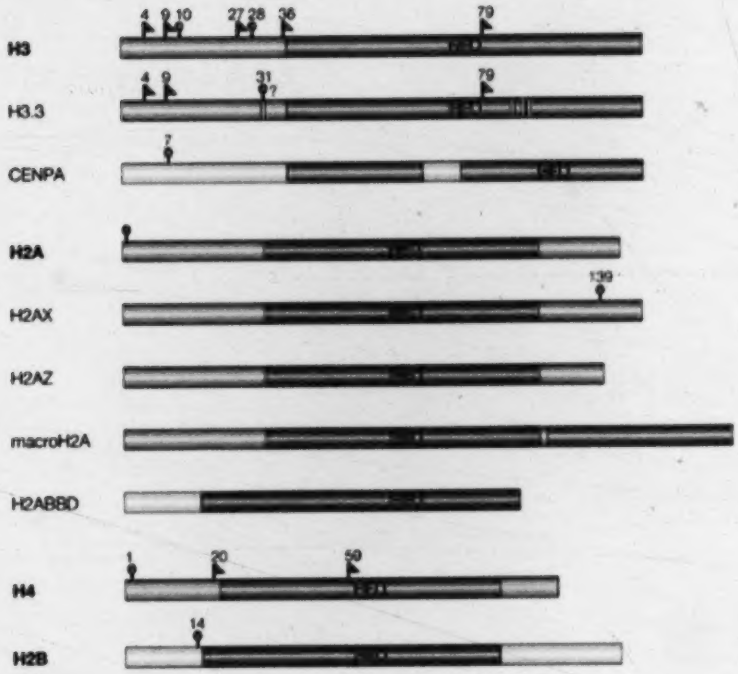


**Figure 1.3. Core histones and their variants.** Each of the core histones (H2A, H2B, H3 and H4) contains a histone fold domain (HFD) and an N-terminal tail that includes sites for various post-translational modifications. Sites of lysine methylation (red flags) and serine phosphorylation (green circles) are indicated. The residues in H3.3 that differ from the core histone H3 (also known as H3.1) are shown in yellow. H2A.Z differs significantly from core H2A in its C-terminus. H2A.X harbors a conserved serine residue (Ser139) which is phosphorylated in response to DNA DSBs. Although budding yeast lacks H3.3 and H2A.X variants, core H3 is most similar to H3.3 and core H2A is phosphorylated on a serine residue in the C-terminus following DNA damage. Figure is adapted from Sarma and Reinberg, 2005.

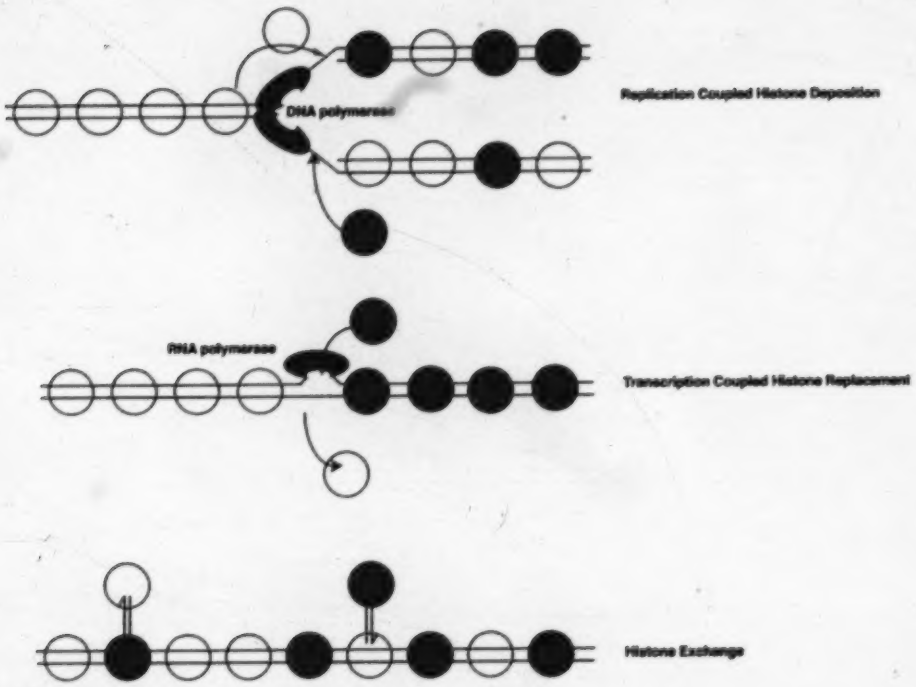


**Figure 1.4. A schematic diagram of the different modes of variant nucleosome incorporation into chromatin.** Variant histones can be incorporated into chromatin in three manners: 1) Replication-dependent chromatin assembly, 2) Transcription-coupled histone replacement, 3) Histone exchange. Variant histone-containing nucleosomes are shown in red. Figure is from Kamakaka and Biggins, 2005.

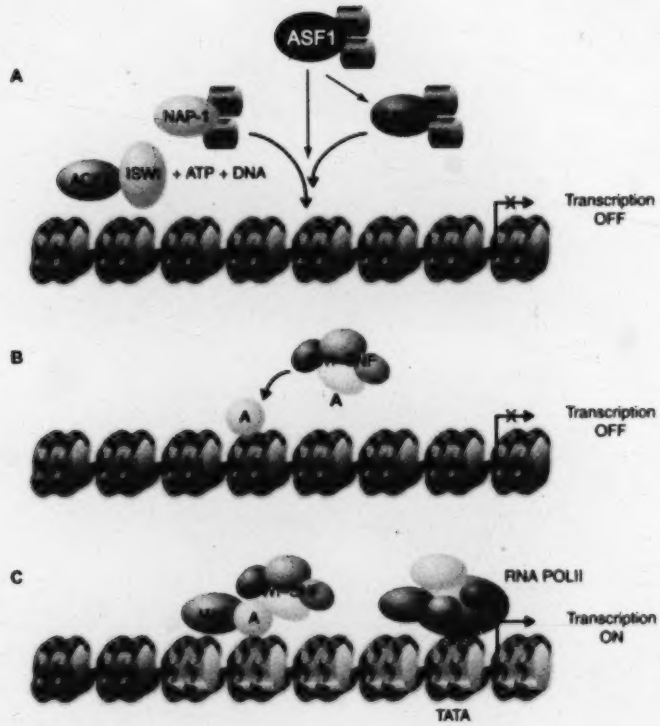


**Figure 1.5. Model of chromatin assembly and transcriptional activation. A.** Following replication, DNA is assembled into chromatin by the combined action of histone chaperones such as Asf1, CAF-1, ACF1 (Acf1 and Isw1), Nap1, and core histones H2A, H2B, H3 and H4. Once assembled, the promoter is transcriptionally inactive. **B.** Interaction with a sequence-specific DNA-binding protein (A) results in recruitment of the SWI/SNF remodeling complex which facilitates the stable association of protein A with the promoter. **C.** After remodeling, a histone acetyltransferase (HAT) is targeted by protein A to the promoter where it acetylates nucleosomes. The combined and sequential action of remodeling and modifying complexes after recruitment by protein A results in a structurally altered chromatin template that can efficiently form a productive transcriptional initiation complex (RNA POLII). Figure is adapted from Kadam and Emerson, 2002.

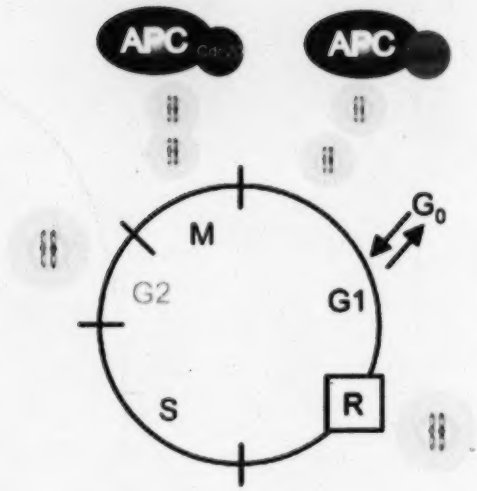


**Figure 1.6. APC-dependent proteolysis during the cell cycle.** The point at which cells reach a threshold cell size in $G_1$ is known as the 'Restriction point' in mammalian cells (R), or 'Start' in budding yeast. At this point of no return the decision is made to complete an entire cell cycle or to exit the cell cycle and enter stationary phase ($G_0$). S phase DNA replication is followed by $G_2$ and then mitosis (M), the phase in which chromosomes are separated. The APC is associated with the Cdc20 activator and functional during M. The APC is associated with the Cdh1 activator and functional during $G_1$. Figure is adapted from Wasch and Engelbert, 2005.

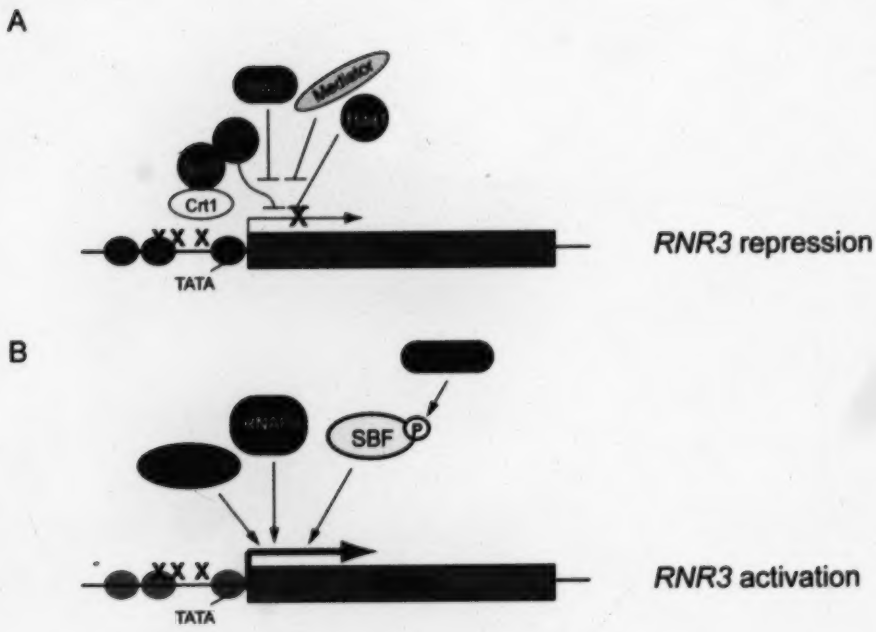


**Figure 1.7. A model of the factors involved in *RNR3* transcriptional regulation. A.** A schematic map of the *RNR3* gene under repressed conditions. Nucleosomes positioned over the promoter are shown as grey circles. Promoter X boxes are indicated by the letter X (in black). Proteins involved in establishment of chromatin structure and *RNR3* repression are indicated. **B.** A map of *RNR3* under inducing conditions when there is a reduction in histone crosslinking to the *RNR3* promoter (Zhang and Reese, 2004a). Shown are the proteins known to be required for chromatin remodeling and transcriptional activation of *RNR3*.

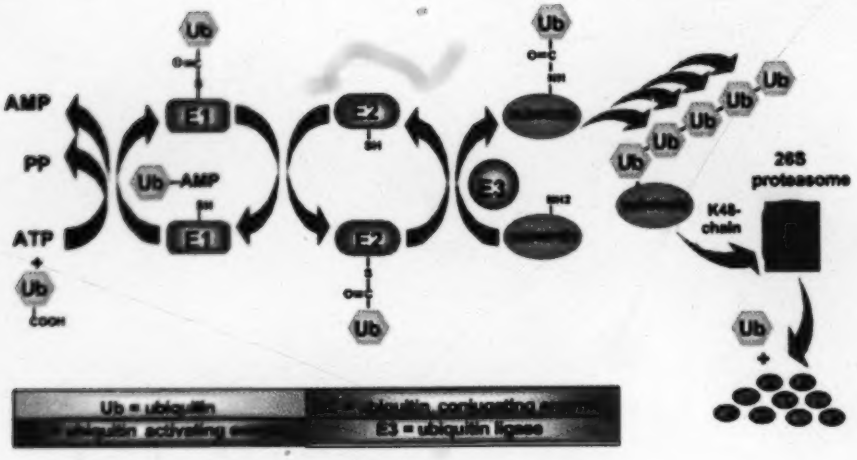


**Figure 1.8. The multi-enzyme cascade of ubiquitin-mediated proteolysis.** In the first reaction, the E1 ubiquitin activating enzyme activates the C-terminus of ubiquitin through formation of an ATP-dependent thioester bond. This is followed by transfer of ubiquitin to an active site cysteine on the ubiquitin conjugating enzyme (E2). The E3 ubiquitin ligase then conjugates ubiquitin to a lysine residue on the substrate protein. Subsequent cycles through this cascade lead to the formation of a lysine 48-linked polyubiquitin chain. This leads to substrate recognition followed by degradation by the 26 S proteasome. Figure is from Pray et al., 2002.

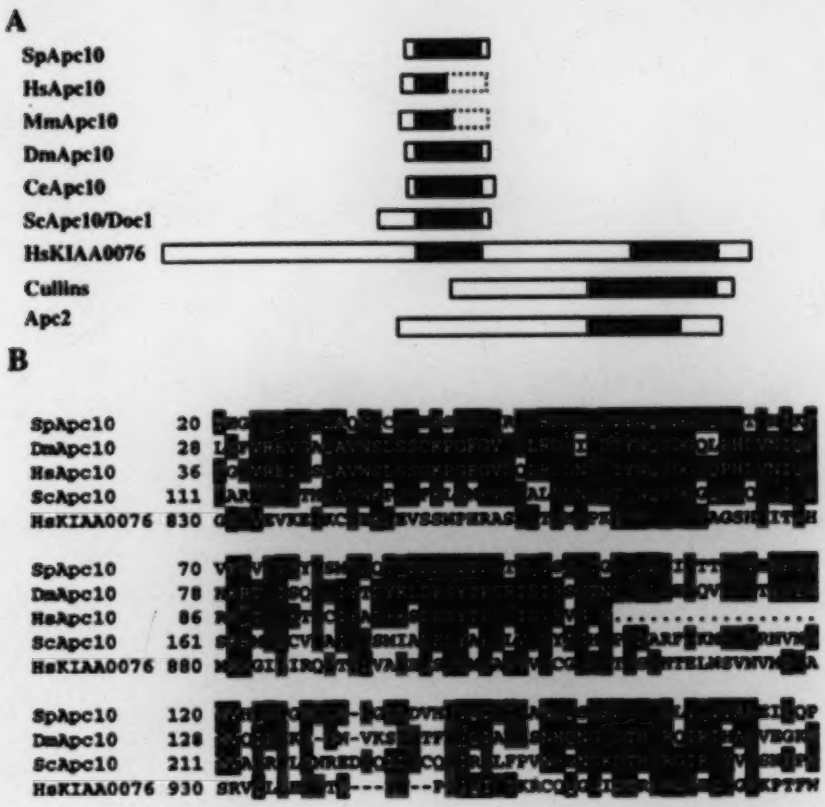


**Figure 1.9. Amino acid sequence comparison of Apc10 between organisms. A.** The overall homology shared between Apc10-related proteins is shown. Species are indicated by the following abbreviations: Sp, fission yeast; Hs, human; Mm, mouse; Dm, fly; Ce, worm; and Sc, budding yeast. A human protein (KIAA0076) which shows homology to both Apc10 (closed box) and the C-terminal region of cullins and Apc2 (shadowed box) is also shown. **B.** The amino acid sequence of the central 150 amino acid residues of Apc10 is compared with that of Apc10 homologues in other species and to the human ORF KIAA0076. Identical amino acids are shown by black boxes with white letters, while shadowed boxes with black letters represent conserved amino acids. Figure is from Kominami et al., 1998.

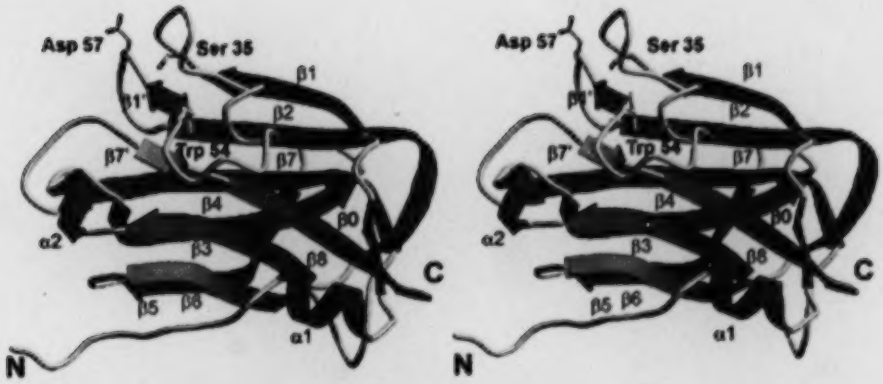


**Figure 1.10. Crystal structure of human Apc10.** Stereo view with labeled secondary structure elements. Color scheme is as follows: The sheets forming both sides of the β-sandwich are colored green and red, α-helices are colored purple, the small β-sheet is colored gray, and the separate β0 strand is colored blue. The β-sandwich jelly roll motif that contains the Doc domain includes the green and red β-sheets, in addition to the small gray β-sheet. The side chains of serine-35, tryptophan-54, and asparagine-57 are indicated as these highly conserved residues interact via hydrogen bonds to stabilize the structure. Figure is from Wendt et al., 2001.

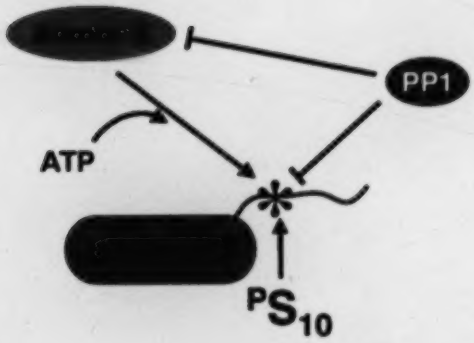


**Figure 1.11. A model for the functional interaction between Aurora B kinase and Protein Phosphatase 1 in H3-S10 phosphorylation.** H3 serine-10 is phosphorylated by Aurora B in mitosis and dephosphorylated by Protein Phosphatase 1 (PP1). PP1 can also dephosphorylate and inactivate Aurora B. Figure is from Prigent and Dimitrov, 2003.

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# Chapter 2:

# Methods and Materials

**Strain growth**

Strains were grown in YPD or selective medium at 30°C and are described in Tables 3.1, 4.1 and 5.1. Deletion mutants were constructed by homologous recombination and one-step disruption using the G418 deletion cassette obtained by PCR from strains generated in the yeast deletion project (Winzeler et al., 1999). The sequences of oligonucleotides used in strain construction are provided in Table 2.1. Correct deletion was confirmed by PCR.

Copper induction of GST plasmids was performed using 0.6 mM $CuSO_4$ for 40-120 min. Treatment with 0.05 or 0.1% methyl methanesulfonate (MMS; Sigma) was for the indicated times; neutralization with 10% sodium thiosulfate was performed for 20 min following treatment (Sidorova and Breeden, 1997). For plating experiments, cultures were inoculated to an $OD_{600}$ of 0.1, grown overnight at 30°C, and 10-fold serial dilutions were plated. For GST-Isw2 overexpression spotting experiments on Ura$^-$ -/+ MMS plates, cultures were inoculated to an $OD_{600}$ of 0.1 and grown at 30°C to an $OD_{600}$ between 0.5 and 1. Copper induction of the plasmid was performed for 40 min and serial dilutions were plated. Plates were photographed after 3-4 d. Expression of GST plasmids was confirmed by anti-GST immunoblotting.

**Yeast transformation**

10 ml cultures were grown to an $OD_{600}$ between 0.2 and 0.7, and cells were collected by centrifugation at 4000 rpm for 5 min. The pellet was washed 1X with 500 µl sterile water and pelleted by centrifugation at 13,000 rpm for 2 min. The pellet was resuspended in 500 µl of 100 mM lithium acetate/1X TE, pH 7.5 and pelleted again. 10 µl carrier DNA (10 mg/ml salmon sperm DNA that had been boiled 5 min and then immediately placed on ice) was added directly to the pellet, followed by addition of the transforming DNA (3-5 µl of plasmid DNA, 20 µl of PCR product for a disruption). 300 µl of Mix (240 µl 50% PEG, 30 µl 1M lithium acetate, 30 µl 10X TE, pH 7.5) was then added and the solution mixed by finger vortexing and using a pipette tip. Following a 30 min incubation at 30°C, the transformation was heat shocked at 42°C for 15 min. The reaction was pelleted and resuspended in 1 ml YPD for a 2 h recovery at 30°C. Following a wash with 100 µl 1 M sorbitol, the final pellet was resuspended in 100 µl 1

M sorbitol and plated on selective medium (5 µl, 10 µl and 50 µl were plated for a plasmid transformation; the entire 100 µl was plated for a disruption). Following 2-3 d growth, the colonies were replica plated onto the same selective medium.

**Genomic DNA isolation**

1X TE (pH 8.0)

10 mM Tris base

1 mM EDTA

DNA lysis buffer

2% Triton X-100

1% SDS

100 mM NaCl

10 mM Tris-Cl (pH 8.0)

1 mM EDTA

Genomic DNA was isolated as described by Hoffman and Winston (1987). 10 ml cultures were grown to saturation, collected by centrifugation for 5 min at 4°C, and washed once with water. The pellet was resuspended in 200 µl DNA lysis buffer by vortexing; 200 µl phenol:$CHCl_3$:IAA (25:24:1) and 0.3 g acid-washed glass beads were added and vortexing was performed for 4 min at 4°C. Following addition of 200 µl TE, the tube was centrifuged for 5 min and the aqueous layer was transferred to a fresh tube. 1 ml 100% ethanol was added, and the tube was mixed by inversion and centrifuged for 2 min. After discarding the supernatant, the pellet was resuspended in 0.4 ml TE plus 3 µl of a 10 mg/ml solution of RNase A. The samples were incubated for 5 min at 37°C and then 10 µl of 4 M ammonium acetate plus 1 ml of 100% ethanol were added and mixing was by inversion. After a 2 min centrifugation, the supernatant was discarded and the pellet was air-dried for at least 2 h and resuspended in 50 µl TE (for PCR analysis) or 300 µl D 5/2 buffer (for Micrococcal Nuclease digestion).

**Release of plasmid for transformation of *E. coli* or yeast**

Plasmid DNA was prepared as described by Hoffman and Winston (1987). 10 ml cultures were grown to saturation, collected by centrifugation for 5 min at 4°C, and washed once with water. The pellet was resuspended in 200 µl DNA lysis buffer by

vortexing; 200 µl phenol:$CHCl_3$:IAA (25:24:1) and 0.3 g acid-washed glass beads were added and vortexing was performed for 2 min at 4°C. The sample was centrifuged for 5 min and the aqueous layer was transferred to a fresh tube. Competent *E. coli* cells were transformed with 1-5 µl of the aqueous layer. Yeast cells were transformed with 100 µl of the aqueous phase.

***E. coli* electroporation**

Electroporation cuvettes, microfuge tubes (for recovery), and the electroporation slide were cooled on ice. 1-2 µl of plasmid DNA was added to a 30 µl aliquot of electrocompetent cells thawed on ice. The mixture was transferred to a 0.1 cm electroporation cuvette, tapped to get all material to the bottom, and placed in the slide. The slide was pushed into the chamber until the cuvette was seated between the contacts and pulsed once at 1.8 kV. The time constant was usually between 4 and 5 msec. The cuvette was removed from the slide, 1 ml LB was added, and the sample was mixed. The tube was then incubated at 37°C for 1 h and 25 µl, 50 µl and 100 µl were plated onto selective medium.

**Cell cycle analysis**

Cells were arrested in $G_1$ with 10 µg/ml α-factor (supplemented at 90 min), S phase with 150 mM hydroxyurea, or $G_2$/M with 20 µg/ml nocodazole. Flow cytometry was performed according to Epstein and Cross (1992) using a FACScan instrument (Becton Dickinson). Cells were fixed in 70% ethanol at 4°C overnight and then incubated in 50 mM Tris-HCl pH 8.0 supplemented with 1mg/ml RNase A at 37°C for at least 2 h. This was followed by a 1 h treatment with 5 mg/ml Pepsin at 37°C and neutralization with 50 mM Tris-HCl pH 8.0. Propidium iodide staining was performed overnight at 4°C and the stained cells were diluted in 50 mM Tris-HCl, pH 8.0 and sonicated immediately prior to analysis.

**Chromatin immunoprecipitation (ChIP)**

Beadbeater lysis buffer

50 mM Hepes, pH 7.5

10 mM $MgCl_2$

150 mM KCl

0.1 mM EDTA

10% glycerol

0.1% NP-40

1 mM DTT

1 mM sodium metabisulfate

0.2 mM PMSF

1 mM benzamidine

1 μg/ml pepstatin

IP buffer

25 mM Hepes, pH 7.5

150 mM KCl

1 mM EDTA

12.5 mM $MgCl_2$

0.1% NP-40

1 mM sodium metabisulfate

0.2 mM PMSF

1 mM benzamidine

1 μg/ml pepstatin

IP elution buffer

50 mM Tris/HCl, pH 8.0

10 mM EDTA, pH 8.0

1% SDS

50X TAE (1L)

242 g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

5X TBE (1L)

54 g Tris base

27.5 g boric acid

20 ml 0.5 M EDT (pH 8.0)

Extracts were prepared as described by Hecht and Grunstein (1999). Cells from a 200 ml culture at $OD_{600}$ = 1.0 -1.2 were crosslinked with 1% formaldehyde for 25 min at 30°C, and then 125 mM glycine was added for an additional 5 min incubation. MMS-treated samples were neutralized with 10% sodium thiosulfate, pelleted, and resuspended in fresh medium prior to crosslinking and lysis. The cell pellets were washed twice with PBS and once with ChIP lysis buffer. 1 ml of the resuspended cells was combined with 1 ml of 0.5 mm glass beads and lysis was performed using a bead beater with four 1 min sessions separated by 2 min on ice between each session. The extract was recovered by passing the bead/extract slurry through a 5 ml syringe fitted with a needle. Purified chromatin was sonicated for 3 x 40 s cycles with a Branson sonicator at 50% output to yield DNA fragments of ≈ 500 bp. The extract was spun twice at full speed for 5 min at 4°C to remove cell debris and protein concentration was determined.

Immunoprecipitations using 1 mg of protein were performed with 12CA5 (Roche), rabbit IgG (Sigma), or an antibody to histone H3 (Abcam # ab1791) overnight at 4°C. Prewashed beads were then added for 2 h at 4°C, followed by 4 x 15 min washes with ChIP IP buffer. The immunoprecipitated material was eluted from the beads by adding 100 µl ChIP IP elution buffer and performing 2 x 30 min incubations at 65°C. Crosslink reversal of the combined eluates overnight at 65°C was followed by DNA purification. The DNA was treated with 100 µg/ml proteinase K for 2 h at 37°C, extracted with phenol/chloroform, precipitated, and then treated with 100 µg/ml RNase A for 30 min at 37°C. The DNA was PCR-amplified for 30 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 2 min, and products were separated on a 1% agarose gel. Primer sequences are listed in Table 2.2.

Quantitative PCR: Primers were of $T_m \approx 60°C$ (Tables 2.2 and 2.3). DNA was analyzed by PCR performed in the linear range using $^{32}$P-dCTP incorporation, 10% TBE-polyacrylamide gel electrophoresis and phosphorimager quantitation of radioactive bands in dried gels. The signal corresponding to the immunoprecipitated product was normalized to the PCR signal derived for the input material (using a 1:100 to 1:1000 dilution).

**Chromatin structure and Southern blotting analysis**

S buffer

1.4 M sorbitol

40 mM Hepes, pH 7.5

0.5 mM $MgCl_2$

F buffer

18% (w/v) Ficoll 400

20 mM PIPES, pH 6.5

0.5 mM $MgCl_2$

GF buffer

20% (w/v) Ficoll 400

20 mM PIPES, pH 6.5

0.5 mM $MgCl_2$

D buffer

10 mM Hepes, pH 7.5

0.5 mM $MgCl_2$

0.05 mM $CaCl_2$

D 5/2 buffer

10 mM Hepes, pH 7.5

5 mM $MgCl_2$

2 mM $CaCl_2$

MNase Stop buffer

5% SDS

5 mg/ml proteinase K

Prehybridization/Hybridization Solution

0.525 M $Na_2HPO_4 \cdot H_20$, pH 7.2

7% SDS

1 mM EDTA, pH 8.0

1 % BSA

Wash buffer 1

0.04 M $Na_2HPO_4 \cdot H_20$, pH 7.2

5% SDS

1 mM EDTA, pH 8.0

Wash buffer 2

0.04 M $Na_2HPO_4 \cdot H_20$, pH 7.2

1% SDS

1 mM EDTA, pH 8.0

Wash buffer 3

0.2X SSC

0.1% SDS

Nuclei preparation and nucleosome mapping was performed as in Ryan et al., 1999. 1 L wild type and *asf1*Δ cells were grown to an $OD_{600}$ of 1, harvested by centrifugation for 5 min at 4500 rpm at 4°C, and washed twice with S buffer containing 10 mM β-ME and 1 mM PMSF. Cells were spheroplasted in the same buffer supplemented with 10 mg of Zymolyase 100T (1 ml of a 10 mg/ml resuspension in 1 M sorbitol) and incubated at 30°C with gentle shaking (60 rpm) for 30-90 min. The extent of spheroplasting was monitored by microscopic analysis. The enzyme was then washed out and the pellet was resuspended in F buffer containing 1 mM PMSF, transferred to a homogenizer and dounced on ice 10 strokes. This lysate was gently layered on top of an equal volume of GF buffer containing 1 mM PMSF and centrifuged at 20,000g in a JA 13.1 swinging-bucket rotor for 30 min at 4°C. The pellet was resuspended in F buffer and vortexed for 5 min in the cold room. Following a 15 min centrifugation at 3000g, the supernatant was transferred to a clean tube and centrifuged at 20,000g for 30 min at 4°C. The pellet, which contained nuclei, was washed once with 10 ml D buffer. 100 µl of sample was removed and the $OD_{600}$ was measured in order to equalize final resuspension volumes between samples based on pellet size. The final pellet was resuspended in 2-4 ml of D buffer. Micrococcal nuclease (MNase) digestion of a 300 µL aliquot was performed with 0, 0.125, 0.25, and 0.5 U/ml of MNase (Sigma) for 10 min at 37°C. Naked DNA samples that had been resuspended in 300 µl D 5/2 buffer were digested in parallel with 0.15 and 0.2 U/ml MNase. The reaction was stopped by adding 55 µl of Stop buffer and incubation at 37°C overnight, extracted with an equal volume of phenol/chloroform and then chloroform, and the DNA was precipitated and resuspended

in 100 µl TE/RNase A. The digested naked DNA samples were treated with Stop buffer for 30 min at 37°C before DNA extraction and precipitation. 30 µl of the DNA was digested with *Pst*I, purified, and then run on a 1.2% agarose-TAE gel and transferred to a nylon membrane for Southern blotting. Hybridization at 65°C for 16 h was to the PCR-amplified random-labeled *Pst*I probe for *RNR3* (Li and Reese, 2001).

**Microarray and Northern blotting analysis**

AE buffer

50 mM NaOAc, pH 5.3

10 mM EDTA

20X Borate Buffer (pH 8.3)

0.4 M boric acid

4 mM EDTA

20X SSC (pH 7.0)

3 M NaCl

0.3 M sodium citrate

Prehybridization/Hybridization Solution

50X Denhardt's solution

5X SSC

1% SDS

10 mg/ml herring sperm DNA

Wash buffer 1

2X SSC

0.1% SDS

Wash buffer 2

0.1X SSC

0.1% SDS

Total RNA was isolated in parallel from BY4741 and deletion mutant strains, grown to an $OD_{600}$ of 0.5 or stationary phase, by hot phenol extraction according to Ausubel et al. (1995). Following a 5 min 4500 rpm centrifugation at 4°C, the pellet was resuspended in water (5X the pellet volume), pelleted and flash-frozen in liquid nitrogen. For a 10 ml starting culture volume, the pellet was resuspended in 400 µl AE buffer and

40 µl 10% SDS by vortexing. 400 µl AE-buffered phenol was added and the solution was incubated at 65°C for 5 min, vortexing at every 1 min interval. The tubes were then flash-frozen in a dry ice/95% ethanol bath for 10 s and then centrifuged for 5 min at RT at 13,000 rpm. The aqueous phase was transferred to a new microfuge tube and 400 µl AE-buffered phenol/$CHCl_3$/IAA (25:24:1) was added. Following a 20 s vortex and 5 min RT spin, the aqueous phase was transferred to a new tube and the RNA was precipitated by addition of 2.5 volumes of 100% ethanol and 40 µl 3 M NaOAc, pH 5.3, and incubated for at least 1 h at -20°C. The samples were spun down at 4°C for 20 min, washed 1X with 70% ethanol, and the pellet was air dried for at least 2 h. The RNA was then resuspended in 20 µl DEPC-treated water and its concentration was measured.

mRNA and then biotin-labeled target cRNA was prepared and hybridized to Affymetrix yeast S98 whole genome oligonucleotide microarrays (all procedures according to manufacturer's instructions). MIAME compliant protocols were followed and are available at www.biochem.ualberta.ca/SchultzLab/. Chips were processed and data was collected using Affymetrix GeneChip system hardware running Microarray Suite 4.0 software. GeneSpring 4.1.5 was used for refined data analysis. Two independent experiments were performed for each wild type versus mutant strain comparison; fold change for each gene (Appendix Tables 7.1 to 7.6) is the average of the two independent measurements.

For Northern blotting, total RNA was isolated and 10-30 µg was resolved in a 0.8% formaldehyde-agarose gel (0.56g agarose, 3.5 ml 20X borate buffer, 60.2 ml water, 6.4 ml formaldehyde). 5 µl of RNA diluted in water was added to 15 µl sample buffer (900 µl formamide, 300 µl formaldehyde, 180 µl 20X borate buffer) and incubated at 65°C for 15 min prior to gel loading. The gel was run for 3 h at 70V in 1X borate buffer and the RNA was then transferred to an Osmonics nylon membrane by capillary action overnight in 20X SSC. Fragments consisting of the open reading frame of genes were used as probes (sequences are listed in Table 2.4); they were prepared by PCR amplification from genomic DNA followed by random priming (Invitrogen). 12.5 µl of a 50 µl sample of Qiagen-purified PCR product was diluted with 9.5 µl water, boiled for 10 min, and then cooled on ice. This denatured DNA was added to a mix consisting of 2 µl

dATP, 2 µl dGTP, 2 µl dTTP, 15 µl random primers buffer mixture and 5 µl $\alpha$-$^{32}$P-dCTP. 1 µl Klenow polymerase was added and the reaction was incubated at 30°C for 30 min. 5 µl Stop buffer, 5 µl 3M NaOAc, pH 5.2, 2 µl of 10 mg/ml salmon sperm DNA and 2.5 volumes of 100% ethanol were added and precipitation was at -20°C for 1h. The pellet was spun down, washed 2X with 70% ethanol, air-dried, and resuspended in 100 µl water plus 100 µl of 10 mg/ml salmon sperm DNA. Following boiling for 10 min, the denatured probe was added to the UV-crosslinked membrane which had been prehybridized for 30 min at 65°C. Hybridization was for 16 h at 65°C. The membrane was washed twice with wash buffer 1 at room temperature for 10 min each, followed by a 30 min wash with wash buffer 2 at 65°C. For quantitation, signals were captured using a Typhoon Phosphorimager (AP Biotech) and analyzed with ImageQuant 2.0 software. The quantitative values presented in this study represent the average result from 3-5 independent experiments; results between replicates varied by less than 10%. The blots were stripped by incubation with 50% formamide, 10% 0.5 M sodium phosphate, pH 6.5 for 2 h at 65°C. Two washes for 15 min each with wash buffer 2 at 65°C were performed, and the membrane was prehybridized and then hybridized with the new probe as described above.

**Immunoblotting and Immunoprecipitation**

4X SDS-PAGE Lower Buffer (pH 8.8)

0.5 M Tris base

0.4 % SDS

4X SDS-PAGE Upper Buffer (pH 6.8)

0.5 M Tris base

0.4 % SDS

5X SDS-PAGE loading/sample buffer (24 mL)

7 mL 100% glycerol

11.5 mL 20% SDS

6.3 mL 1M Tris/HCl (pH 6.8)

1 g DTT

0.1 g BPB

Semi-dry transfer buffer (pH 8.2)

25 mM Tris base

190 mM glycine

20% methanol

10X TBS (pH 7.5)

100 mM Tris base

150 mM NaCl

5 mM $MgCl_2$

1X TBST

1X TBS, pH 7.5

0.1% Tween-20

1X TBS-Triton

1X TBS, pH 7.5

0.1% Triton-X100

10X SDS-PAGE running buffer (1L)

30 g Tris base

144 g glycine

10 g SDS

For routine immunoblotting, cells were grown to the desired optical density and the equivalent of 10 ml of cells at $OD_{600}$ = 0.5 was collected by centrifugation for 5 min at 4500 rpm at 4°C, washed once with 500 µl water, and then pelleted again. Cells were lysed in 0.5% βME and 0.3 M NaOH for 10 min on ice. Protein was then precipitated with 25% trichloroacetic acid for 10 min on ice. Following a 10 min spin at RT, the pellet was washed twice with 500 µl acetone and air-dried for 10 min. The cells were resuspended in 100 µl SDS-PAGE sample buffer by vigorous vortexing and using a pipette tip. After incubation at 65°C for 10 min and vigorous vortexing, proteins were resolved in 6-15% SDS polyacrylamide gels and electroblotted to nitrocellulose membranes. Following blocking, membranes were incubated with primary antibody (in 4% BSA, TBST or 3% milk powder/TBST). Washes were routinely performed using TBST, with the exception of TAP-tagged protein western blotting in which washes were performed using TBS-Triton. This was followed by detection using horseradish peroxidase-conjugated goat anti-rabbit (1:20,000), rabbit anti-goat (1:2000) or goat anti-

mouse secondary antibody (1:5000 dilution) and enhanced chemiluminescence (AP Biotech). Primary antibody dilutions were: anti-HA (12CA5; Roche), 1:1000; anti-CBP (Upstate), 1:5000; anti-GST (Amersham), 1:2000; anti-actin (Chemicon), 1:1000; anti-Rad53 (Santa Cruz), 1:1000; anti-TBP, 1:5000 (Ghavidel and Schultz, 2001); H2B antiserum, 1:20,000; affinity purified anti-H3 IgG, 1:200; H4 antiserum, 1:20,000; K9/K14 di-acetylated H3 IgG (Upstate), 1:3000; K5/K9/K12/K14 tetra-acetylated H4 IgG (Upstate), 1:1000; phospho-S10 H3 IgG (Upstate), 1:1000; K4 di-methylated H3 IgG (Upstate), 1:2000; full length H4 IgG (Upstate), 1:1000. Quantitation of immunoblots was performed by optical scanning using a Fluor-S Multiimager (Bio-Rad) and Quantity One v. 4.2.3. software according to the manufacturer's instructions.

**Coimmunoprecipitation and immunoblotting**

N-150 lysis buffer

30 mM Hepes, pH 7.9

2 mM EDTA

2 mM EGTA

150 mM KCl

5% glycerol

0.01% NP-40

1 mM DTT

0.2 mM PMSF

2 μg/ml leupeptin

3.5 μg/ml pepstatin

10 μg/ml aprotinin

25 μg/ml TPCK

Log phase cells (100 ml cultures) were subjected to bead beating as in Ghavidel and Schultz, 2001. Briefly, the cells were harvested at an $OD_{600}$ of 0.5-1 and washed once with water. Approximately 100 μl of packed cells were resuspended in 2 volumes of N-150 lysis buffer. Immunoprecipitations were performed using 1 mg of protein. For anti-HA immunoprecipitations, samples were rotated overnight at 4°C with 50 μL of antibody. Protein G Sepharose was then added for 2 h, and beads were washed (4 x 15 min with N-150 buffer). Immunoprecipitation from strains expressing $Cu^{2+}$-induced GST

or GST-Asf1 (Martzen et al., 1999) was performed using glutathione sepharose beads (30 µL of 1:1 slurry) with rotation overnight at 4°C. For anti-TAP immunoprecipitations, samples were rotated overnight at 4°C with 50 µL of a 1:1 slurry of Rabbit IgG Agarose (Sigma). Anti-Tup1 immunoprecipitations were performed using a 1:100 dilution of the antibody (Watson et al., 2000). Following washes, immunoblotting was performed using anti-GST antibody (Amersham, 1:2000) or antibodies to Ssn6 or Tup1 (1:2000; Watson et al., 2000). For protein detection, 2% of input or supernatant and 50% of the precipitate were resolved on 10-15% SDS polyacrylamide gels and electroblotted to nitrocellulose. Incubation with primary antibody (in 4% BSA, TBST or 3% skim milk powder) was followed by detection using horseradish peroxidase-conjugated goat anti-mouse (1:5000), goat anti-rabbit (1:20,000) or rabbit anti-goat secondary antibody (1:2000) and enhanced chemiluminescence (AP Biotech).

For anti-HA immunoprecipitations of ChIP extracts, samples were rotated overnight at 4°C with 10 µL of antibody. Protein G Sepharose was then added for 2 h, and beads were washed (4 x 15 min with ChIP IP buffer). For protein detection, 2% of input or supernatant and 20% of the precipitate were resolved on a 12% SDS polyacrylamide gel and electroblotted to nitrocellulose. Incubation with anti-HA antibody (in 4% BSA, TBST) was followed by detection using horseradish peroxidase-conjugated goat anti-mouse (1:5000) antibody and enhanced chemiluminescence (AP Biotech).

**Preparation and Assay of Plasmid Supercoiling Extracts**

Extracts were prepared as in Schultz (1999) from cells grown to stationary phase in 500 ml YPD. 20 µl assembly reactions were performed for 30 min at 30°C using relaxed $^{32}$P-labeled pBluescript template. Supercoiling was analyzed by agarose gel electrophoresis at 4°C in TAE running buffer.

**Antibodies**

Fusions of residues 1-30 of yeast H3, 1-35 of yeast H2B and 1-30 of yeast H4 with glutathione S-transferase (plasmids from M. Grunstein; Hecht et al., 1995) were produced in *E. coli* and used to immunize New Zealand white rabbits (Altheim and Schultz, 1999). Crude serum was used for H2B and H4 immunoblotting (H193 and H196 respectively). Anti-H3 IgG (from serum H195) was affinity purified using a fusion of

residues 1-63 of yeast H3 to *E. coli* anthranilate synthase (Robinson et al., 1991). Other anti-histone antibodies were from Upstate Biotechnology.

**Table 2.1.** Oligonucleotides used in deletion strain construction

| Gene | Primer 1 Sequence (300-400 bp upstream of gene) | Primer 2 Sequence (300-400 bp downstream of gene) |
|---|---|---|
| *APC10* | CATCTACCCACTTGCGCTTGA TGAAATTTT | GTTAAGCTTCCAAAGTAAACAA CCGAATCC |
| *ASF1* | GTGCCACACCTAACCTTCG | TATTAGGGCGTGTGGCGTA |
| *BDF1* | GCAGTTGCTATTAGCCGC | GATGGGCGTTCTGAGAGA |
| *CRT1* | TCCTATCTGGTACGGTGC | GAAGACGGTGATTCTGAGG |
| *DST1* | GTGACTCGGTTCTCTTAGG | CTTTAGTTCTGACCGAGCC |
| *HTZ1* | AAGCGGTAGTAAGCACATAC | TTAGAAATGGTAGGGGGG |
| *ISW2* | ACGGGCGCTTCTTGTAAT | GGCTCGACCCAGTTAGACTTA |
| *SPT4* | GGGTGAAGAGCTTCCAGAAT | GTGAGGCTTCATGCACTG |
| *YOX1* | TACAGCTCGCTATAGTCGCC | TAACATCACTCAACCGGGGT |

**Table 2.2.** Oligonucleotides used in ChIP analysis

| Gene/ChIP primer set location[a] | ChIP Primer 1 Sequence | ChIP Primer 2 Sequence |
|---|---|---|
| *RNR2*/PR | GATATCACCACCAATTGGC | GCGCGATGTCAAGTAA |
| *RNR3*/PR | TAGGTAGCAGAGCAAGCC | CTGCTGCTATTCTTGCTTGT |
| *RNR4*/PR | TAGACGACGTCCGTTCCTT | GCGGGGTTGTGTGGCTCA |
| *RNR3*/DN | CCTTATCTAACTTGGAGCGG | CTCCCTTCTTCATCAAGC |
| *CRT1*/PR | AGCAGTCGACGCATACTTGT | CCAAATCGCCATGACAACA |
| *SAS10*/PR | AGCGCCGGTTACTCTTTT | GCTATGATGAGATGAGC |
| *ACT1*/PR | CCATTTTCTTCTTTACCCGCC | TCAGTAAATTTTCGATCTTGGG |
| *CUP1*/PR | CCCCGTTAGTGAACTGAAC | CTGACAATCCATATTGCG |
| *HHF2*/PR | CTGGCCATTGTGGAGTGTT | AGGCGCGAGTGAACAACAT |
| *HTA1*/PR | GCCCCTTTCTTACCAATCG | TTACGATCCACTGGCTG |
| *HHT1*/PR | CAATGAATGGGGAAGGGG | GAGGAAAGAGATGTATCCCG |
| *ADH1*/PR | GGTATACGGCCTTCCTTCCAGTTAC | GAACGAGAACAATGACGAGG<br>AAACAAAAG |
| *SED1*/PR | TTTAGATTGGCCGTAGGGGC | CAAGAGAATAGAAAAAGAGA<br>GGTGAG |
| *PGK1*/PR | CATCTAAGAACTTGAAAAACTACG | CAGCCTGTTCTCACACACTC |
| *PPH3*/PR | AGATCGGCTTCTTACACC | CGTGGACTAGCGTGTGAC |
| *RPR1*/PR | AGGGCAAATGTACTCTCG | AATCGCAGCTCCCAGAG T |
| *HSP30*/PR | TTAGTGCCGCGTGATACC | CTTGTCCTTGCCATAGCAG |
| *DDR2*/PR | CCTCTAGAGAAAACCGGCA | TTAGCGTGCTTGCTTGGA |
| MFα2/PR | TCTACATCATTACCCCGC | ATTAGCGTGCTTGCTTGGAG |
| *GPH1*/PR | CGGGAGTGTCCTACTGCT | AAGAAACGGGAAGGCAC |
| Chromosome V | GGCTGTCAGAATATGGGGCCGTAGTA | CACCCCGAAGCTGCTTTCACAATAC |

[a]PR, promoter; DN, downstream of coding region.

**Table 2.3.** Oligonucleotides used in *RNR3* ChIP analysis

| Primer Set | ChIP Primer 1 Sequence | ChIP Primer 2 Sequence |
|---|---|---|
| PR1 | CTATCCGCAGCAGCTGGA | AACTAGCCACGGCCACAG |
| PR2 | GTTATCTGCCTACGGTTG | GCTGCTGCTATTCTTGCTTGTC |
| CD1 | ACCACTGTGCACCCTGATT | GACATTTGAGGATGTGGCG |
| CD2 | GATGATGTGTGGGGTGATG | TAGCCTCGGGAATGGATA |
| CD3 | ACCGTCTGGGAAATCTCTC | GCTGGGGCAACACTATCTT |

**Table 2.4.** Oligonucleotides used in Northern blotting

| Gene | Forward Oligonucletide Sequence | Reverse Oligonucleotide Sequence |
|---|---|---|
| *RNR3* | CTCCCGTATCACCCGTT | CGATATCGCTACCATGG |
| *RNR2* | CGCTTCTGACGGTATTGT | GGCGTCCAAGAAGTATCT |
| *HUG1* | AAGGCCTTAACCCAAAGC | CAATGTCAGAAAGACCGC |
| *UBI4* | GGTTGAATCCTCCGACAC | GAAGATTCAACCTCTAGGG |
| *ACT1* | GCTCAATCCAAGAGAGG | CCAAGGCGACGTAACATAG |
| *GLC7* | TGTGGTGACATTCATGGG | CTTCCACAACTTGATGGG |

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**Part I:**

**Chapter 3:**

# Transcriptional regulation of a DNA damage response gene by dynamic targeting of histone chaperone Asf1

**Summary:**

Histone-binding chaperone Asf1 is activated by DNA damage signals for chromatin assembly at sites of DNA repair. Our in vivo analysis of gene expression and chromatin metabolism reveals that yeast Asf1 also functions in a targeted pathway of nucleosome assembly/stabilization which regulates transcription of a DNA damage inducible gene, *RNR3*. Under benign conditions Asf1 is specifically localized to *RNR3*, where it represses transcription by contributing to histone association with the promoter. DNA damage signals trigger dramatic dissociation of Asf1 from *RNR3*. This dissociation occurs in concert with reconfiguration of promoter nucleosomes and transcriptional induction but it is not caused by transcription. In *asf1Δ* cells *RNR3* is derepressed and promoter chromatin is destabilized; exposure to genotoxin triggers further disruption of *RNR3* chromatin structure and hyperinduction of transcription. Collectively these findings reveal a new mechanism of gene regulation in which reversible, targeted association of a histone chaperone functions in localized assembly and/or stabilization of repressive nucleosomes.

**Introduction:**

CAF-1 and Asf1 are highly conserved chaperones which bind to histones H3 and H4. The role of CAF-1 in replication and repair-coupled chromatin assembly has been well-established (Smith and Stillman, 1989; Gaillard et al., 1996; Moggs et al., 2000; Kaufman et al., 1995). The CAF-1 complex is composed of three subunits, Cac1, Cac2 and Msi1 (Cac3), and is targeted to replication forks through an interaction between the Cac1 subunit and the processivity clamp, proliferating cell nuclear antigen (PCNA) (Shibahara and Stillman, 1999; Moggs et al., 2000). The human Cac2 subunit interacts physically with Asf1 (Mello et al., 2002). Based on the close sequence homology to a HAT complex component and tight association with a histone deacetylase complex in humans, it has been proposed that the Msi1 subunit may have additional roles independent of its involvement in the CAF-1 assembly complex (Parthun et al., 1996; Taunton et al., 1996; Verreault et al., 1996). Msi1 is also an antagonist of the Ras-cyclic AMP signaling pathway (Ruggieri et al., 1989), further establishing its functional independence from Cac1 and Cac2. Although none of the CAC genes is required for viability, their individual deletion results in increased sensitivity to UV radiation and disruption of telomeric silencing (Enomoto and Berman, 1998; Enomoto et al., 1997; Game and Kaufman, 1999; Kaufman et al., 1997; Monson et al., 1997). Asf1 and the CAC genes interact genetically, however, the phenotypes of an Asf1 mutant alone are much more pronounced and include sensitivity to DNA damaging agents that cause single and double-strand breaks, and to the replication inhibitor, hydroxyurea (Tyler et al., 1999).

In addition to its involvement in replication-coupled chromatin assembly, Asf1 also performs replication-independent chromatin assembly (Mello et al., 2002; Munakata et al., 2000; Robinson and Schultz, 2003; Tyler et al., 1999). Various functions of Asf1, for example in DNA repair (Emili et al., 2001; Hu et al., 2001; Mello et al., 2002; Tyler et al., 1999), the maintenance of genome stability (Myung et al., 2003; Prado et al., 2004), and transcriptional regulation (Krawitz et al., 2002; Meijsing and Ehrenhofer-Murray, 2001; Osada et al., 2001; Sharp et al., 2001; Sutton et al., 2001), are thought to largely depend on its participation in de novo nucleosome assembly. In other words, Asf1 can indirectly modulate DNA-dependent processes in the nucleus by virtue of its

capacity to assemble nucleosomes. The global effects of Asf1 on such processes as transcription and the maintenance of genome stability, however, may not be limited to its participation in nucleosome assembly. Most strikingly, recent studies in yeast suggest that Asf1 disassembles nucleosomes on a genome-wide scale; at some genes Asf1-dependent nucleosome disassembly evidently promotes transcriptional activation (Adkins and Tyler, 2004, Adkins et al., 2004).

Beyond its role in bulk chromatin assembly and disassembly, Asf1 may participate in targeted chromatin remodeling events which affect transcription. The observations that Asf1 interacts with core components of the transcriptional machinery (Chimura et al., 2002) as well as chromatin remodeling proteins (Moshkin et al., 2002; Krogan et al., 2003) raise the possibility that Asf1 directly participates in targeted chromatin metabolism by mechanisms which are independent of its global functions in chromatin regulation. The physiological significance of widespread Asf1 localization on chromosomes (Moshkin et al., 2002; Tyler et al., 2001), however, has not been clarified and its mechanism of gene-specific effects on transcription remains to be analyzed in detail. Furthermore, there is no direct evidence that association of Asf1 with chromatin remodeling proteins affects nucleosome remodeling.

The local configuration of chromatin may change in the course of the activation and repression of a gene. If Asf1 promotes chromatin remodeling at specific genes, then one or more of its activities may be regulated during the transcription cycle. Although not characterized in the context of transcriptional regulation, dynamic behavior of yeast Asf1 has been described with respect to its role in chromatin assembly coupled to DNA repair. Under normal conditions a substantial proportion of soluble Asf1 exists in a stable complex which includes Rad53, a protein kinase involved in DNA damage checkpoint signaling (Emili et al., 2001; Hu et al., 2001; Schwartz et al., 2003). Asf1 in complex with Rad53 is unable to participate in chromatin assembly. When cells experience genotoxic stress Asf1 is released from the Rad53 complex and becomes competent for nucleosome assembly coupled to DNA repair (Emili et al., 2001; Hu et al., 2001).

Considering the evidence that Asf1 might directly control transcription and that it can be regulated by genotoxic stress, we were intrigued to find in a microarray

experiment that a small subset of DNA damage response genes is induced in an *ASF1* null strain and CAF-1 mutants that are null for the *CAC1* or *MSI1* subunit. Transcriptional regulation of one of these genes, *RNR3*, has been particularly well-studied. *RNR3* encodes a subunit of ribonucleotide reductase (Rnr), the enzyme which catalyzes the rate-limiting step in dNTP synthesis (Ho et al., 1997; Huang et al., 1998; Li and Reese, 2001; Sharma et al., 2003; Zhang and Reese, 2004a, b). Induction of *RNR3* mRNA in response to DNA damage is thought to promote elevated dNTP synthesis and therefore facilitate DNA repair. This induction involves dramatic alterations of the chromatin structure of *RNR3* controlled in part by the ISW2 chromatin remodeling complex, the Hda1 histone deacetylase, and subunits of TFIID (Li and Reese, 2000; Zhang and Reese, 2004a, b). We hypothesized that Asf1 might also function directly in transcriptional regulation of *RNR3* by DNA damage signals. Testing of this hypothesis revealed that *RNR3* is a direct target of transcriptional regulation by Asf1. Repression of *RNR3* under benign conditions partly involves stable promoter association of Asf1, which contributes to the organization of the repressive state of chromatin at *RNR3*. DNA damage signals trigger release of Asf1 from *RNR3* concomitant with chromatin remodeling and transcriptional derepression.

**Results:**

**Comprehensive repression of DNA damage-inducible genes requires CAFs**

The role of budding yeast CAFs in transcription was initially explored by comparing the mRNA expression profiles of three individual CAF deletion mutants to that of their isogenic wild type partner (Winzeler et al., 1999). Strain genotypes are described in Table 3.1. Total RNA was isolated in parallel from BY4741 and *asf1*Δ, *cac1*Δ, or *msi1*Δ strains grown to an $OD_{600}$ of 0.5. mRNA and then biotin-labeled target cRNA was prepared and hybridized to whole genome oligonucleotide microarrays; the overall results are summarized in Table 3.2. The misregulated genes were located throughout the chromosomes for each of the CAF mutants, and a representative map showing the physical location of all misregulated genes in the *asf1*Δ mutant is shown in Figure 3.1. Upregulated genes for each of the CAF mutants belonged to diverse functional groups, with many involved in regulation of cellular metabolism (Tables 3.3, 3.4, and 3.5). The slow growth of the *asf1*Δ mutant may contribute to the misregulation of this class of genes. However, many genes within this category are also upregulated in the *cac1*Δ and *msi1*Δ strains which are not defective for growth (Kaufman et al., 1997), suggesting that growth rate is not the only factor responsible for misregulation of cellular metabolism genes. Downregulated genes in the *asf1*Δ strain included those encoding ribosomal proteins and Ty transposable elements (Table 3.6). In contrast, genes downregulated in the absence of *CAC1* or *MSI1* included a number of genes involved in RNA processing, as well as genes encoding subunits of RNA polymerases I, II, or III (Tables 3.7 and 3.8). As members of the same chromatin assembly complex, the misregulated genes for the *cac1*Δ and *msi1*Δ datasets showed significant statistical correlation ($r^2 = 0.85$), although there does appear to be some functional independence, as suggested by evidence in the literature (Kleff et al., 1995; Parthun et al., 1996; Taunton et al., 1996; Verreault et al., 1996; Ruggieri et al., 1989). The correlation between transcription profiles for *cac1*Δ and *msi1*Δ when compared to *asf1*Δ are less ($r^2 = 0.53$ and 0.48, respectively), but still suggest a certain degree of functional overlap.

Based on the fact that Asf1 and CAF-1 bind to histones H3 and H4, it was possible that the transcriptional effects observed in the microarray experiment were due to perturbed histone regulation, as changes in histone expression and modification

regulate overall chromatin structure. We addressed this possibility by monitoring whether the levels of bulk and variously modified forms of H3 are altered upon loss of Asf1 by western blotting analysis (Fig. 3.2). The *asf1*Δ strain was chosen for this analysis because it showed the largest degree of transcriptional misregulation. The amino-terminal tail of H3 is post-translationally modified at a number of sites, including acetylation of lysines-9 and -14, methylation of lysine-4 and phosphorylation of serine-10 in budding yeast.

We found no difference in the levels of bulk, S10-phosphorylated, or K4-methylated H3 between wild type and *asf1Δ* cells. Therefore, the altered transcription profile of an *asf1Δ* strain is not due to alterations in the level of H3 expression, or its phosphorylation or methylation. However, upon examination of H3 acetylation in *asf1Δ* cells, it is evident that the level of K9/14 diacetylated H3 was reduced in the mutant (Figure 3.2; compare lane 3 to lane 6). Adkins and Tyler (2004) have shown that this is due to a reduction of H3-K9 acetylation by using an antibody specific for acetylation at this residue. However, these authors found that deletion of the N-terminal tail of H3 did not alter global chromatin structure in an *asf1Δ* strain, and concluded that altered histone modification is not responsible for altering chromatin structure. Combination of the chromatin structure evidence obtained by Adkins and Tyler (2004) and our result showing that this reduction in H3 acetylation is fairly modest in *asf1Δ* cells (Fig. 3.2) suggests that the altered global transcription profile in cells lacking *ASF1* is not fully attributable to any significant alteration of H3 metabolism.

The microarray analysis revealed net overexpression of genes in each of the three CAF mutant strains (Table 3.2), indicating a requirement for Asf1, Cac1 and Msi1 in proper transcriptional repression at numerous loci. Of particular interest was a small group of DNA damage inducible genes that were upregulated; *RNR3*, *RNR2*, *HUG1* and *UBI4* were reproducibly misregulated in the CAF deletion mutants examined by microarray (Table 3.9). This raised the possibility that these CAFs contribute to transcriptional repression at these DNA damage inducible genes. Further experiments focused on *ASF1* as its deletion had a significantly greater effect on transcription than did deletion of either *CAC1* or *MSI1*, and because it has previously been implicated in

transcriptional regulation (Krawitz et al., 2002; Meijsing and Ehrenhofer-Murray, 2001; Osada et al., 2001; Sharp et al., 2001; Sutton et al., 2001).

We began to test the possibility of a direct role for Asf1 in transcriptional regulation by exploring how Asf1 controls transcription of *RNR3*, a gene whose overexpression in *asf1Δ* cells is readily detected by Northern blotting (Fig. 3.3, A; Fig. 3.4, A). The 3.5 to 6.3-fold increase in *RNR3* transcription associated with deletion of *ASF1* (Fig. 3.3, A; Fig. 3.4, A; Chapter 4) falls between the approximately 2-fold 'modest level of derepression' in *ISW2* null mutants lacking an ATP-dependent remodeling enzyme which acts at *RNR3* (Zhang and Reese, 2004a), and the very strong derepression associated with deletion of *CRT1*, which encodes a sequence-specific repressor of *RNR3* (Huang et al., 1998). Accordingly, we judge the effect of *ASF1* deletion to reflect biologically significant regulation of *RNR3* by Asf1.

Various indirect mechanisms have been ruled out as being responsible for *RNR3* induction in the *asf1Δ* mutant. Microarray analysis suggests that misexpression of the known transcriptional regulators (Crt1 and Swi4) of DNA damage response genes is unlikely to account for induction of *RNR3*, *RNR2* or *HUG1* mRNAs when Asf1 is absent (Basrai et al., 1999; Ho et al., 1997; Huang et al., 1998; Li and Reese, 2001). For example, if deletion of *ASF1* had an indirect effect on the expression of *CRT1*, then this could account for induction of these DNA damage response genes. However, expression of *CRT1* mRNA, encoding a transcriptional repressor of *RNR2*, *RNR3* and *HUG1*, is not dampened in *asf1*Δ cells (Appendix Table 7.4). Therefore, induction of these genes is not likely due to decreased synthesis of Crt1. *SWI4*, a gene which encodes a DNA-binding factor implicated in *RNR3* upregulation in response to genotoxic stress (Ho et al., 1997), is 4-fold induced in *asf1*Δ cells (Appendix Table 7.1). In wild type cells, 6- and 4.5-fold transcriptional induction of *SWI4* and *RNR3* accompanies release from $G_1$ arrest (Spellman et al., 1998). However, under these conditions Swi4 is not recruited to the *RNR3* promoter (Horak et al., 2002). Furthermore, *RNR3* transcription in normal cells is not sensitive to deletion of *SWI4* (Ho et al., 1997). We conclude that upregulation of *SWI4* in normally growing *asf1*Δ cells is unlikely to directly underlie derepression of *RNR3*.

Although DNA damage checkpoint signaling is the best characterized mode of *RNR3* induction (Huang et al., 1998), microarray studies have shown that *RNR3* transcriptional regulation can be exerted by other signaling pathways. Since *RNR3* is repressed during the diauxic shift (approximately 3-fold; DeRisi et al., 1997), nutrient sensing pathways may play a role in *RNR3* transcriptional regulation. Clearly *RNR3* is transcriptionally regulated by both checkpoint-dependent as well as checkpoint-independent mechanisms, and the possibility of *RNR3* regulation independent of weak checkpoint activation in *asf1*Δ cells is outlined below.

The DNA damage checkpoint is activated in *asf1*Δ cells as a result of spontaneous DNA damage during replication (Ramey et al., 2004). DNA double strand breaks (DSBs) that occur due to DNA damage or replication fork stalling during S phase are detected by the replication checkpoint which inhibits ongoing DNA synthesis until the damage is repaired (Osborn et al., 2002). The replication checkpoint therefore protects the cell from spontaneous damage that arises during S phase. Since this DNA damage checkpoint is activated in *asf1*Δ cells and their S phase progression is slower, it was possible that overexpression of *RNR3* in the absence of Asf1 could be due to constitutive engagement of a damage-dependent mechanism for increasing transcription of *RNR3* (Huang et al., 1998), and not any effect on an unknown pathway of gene regulation by Asf1. We addressed this possibility in a number of experiments.

If checkpoint activation by replicational stress does underlie *RNR3* induction in an asynchronous culture of *asf1*Δ cells, then in a $G_1$ population of the mutant *RNR3* expression should not be elevated because: 1) cells undergoing replication are not present, 2) having successfully traversed the M-$G_1$ boundary but not entered S phase, *asf1*Δ cells cannot develop replicational stress, and 3) even if *asf1*Δ cells entered $G_1$ with unrepaired DNA because they adapted to the damage, the DNA damage checkpoint will not be engaged (Lupardus and Cimprich, 2004). To test this prediction we first assessed the status of the DNA damage checkpoint in cycling and $G_1$-arrested (Fig. 3.3, B) wild type and *asf1*Δ cells by monitoring Rad53 phosphorylation, which is elevated when the DNA damage checkpoint is engaged (Sanchez et al., 1996; Sun et al., 1996). As expected, a substantial mobility shift of Rad53 characterizes its hyperphosphorylation in MMS-treated wild type cells (Fig. 3.3, C, lanes 1-3). Under normal growing conditions,

deletion of *ASF1* is also associated with a change in the migration pattern of Rad53. Lanes 1 and 4 in Figure 3.3, C shows that Rad53 from wild type cells migrates predominantly as a single band (arrow), which is resolved from a less intense, slower-migrating band (asterisk). An additional population of Rad53 molecules, of slightly lower mobility than the bulk of the Rad53 in wild type cells, is present in *asf1Δ* cells (similar results were reported by Schwartz et al., 2003). Owing to the presence of this possibly hyperphosphorylated form of Rad53, the faster and slower migrating bands detectable in samples from wild type cells (arrow and asterisk in lane 4) are not clearly distinguishable in *asf1Δ* samples (Fig. 3.3, C, lane 5). Whereas Rad53 has a different pattern of migration in wild type and *asf1Δ* cells that are cycling, under conditions of $G_1$ arrest the band pattern is virtually indistinguishable between these strains (Fig. 3.3, C, compare lanes 6, 7). Furthermore, the band pattern in $G_1$-arrested cells is characteristic of cycling wild type cells, in which the DNA damage checkpoint is not engaged. Therefore, Rad53 is not activated in $G_1$-arrested *asf1Δ* cells. Although the DNA damage checkpoint is not engaged, differential expression of *RNR3* is readily apparent between $G_1$-arrested wild type and *asf1*Δ cells (Fig. 3.3, D). We conclude that activation of the DNA damage checkpoint as it occurs in cycling *asf1*Δ cells is unlikely to account for overexpression of *RNR3*. An implication of this finding is that checkpoint activation in response to spontaneous DNA damage in cycling *asf1*Δ cells differs somehow from checkpoint activation which results from treatment with genotoxin and is dependent on the Rad53 and Mec1 checkpoint protein kinases. Consistent with this interpretation, treatment of *asf1*Δ cells with genotoxin induces the appearance of post-translationally modified forms of Rad53 which are completely absent from untreated *asf1*Δ cells (Hu et al., 2001; Ramey et al., 2004). We also find that *RNR3* transcription in *asf1*Δ cells is inducible by treatment with a DNA damaging agent or a replication inhibitor (see below).

*RNR3* expression could be elevated in *asf1*Δ cells as a secondary consequence of their previously documented S phase and $G_2$/M cell cycle progression defects (Emili et al., 2001; Sutton et al., 2001; Tyler et al., 1999). We directly tested if abnormal $G_2$/M progression is responsible for *RNR3* induction in the absence of Asf1 by measuring *RNR3* expression in wild type and null mutant cells arrested in $G_2$/M with nocodazole (Fig. 3.3,

E; the right-shift of the *asf1*Δ $G_2$/M peak is probably due to the larger size of the mutant, as noted by Zhang et al., 2002, and/or the presence of replicated mitochondrial DNA). Figure 3.3, F, shows that induction of *RNR3* is reproduced in $G_2$/M-arrested wild type and *asf1*Δ cells. Therefore, the elevated expression of *RNR3* in mixed cultures of *asf1*Δ cells is not due to the high proportion of $G_2$/M cells in such cultures. Because nocodazole-treated wild type and *asf1*Δ cultures do not contain S phase cells (Fig. 3.3, E) but do differentially express *RNR3*, we can also exclude the possibility that abnormal S phase progression underlies *RNR3* induction in the mutant.

We next examined whether the transcriptional response of *RNR3* to DNA damage is normal in *asf1*Δ cells. If Asf1 has a role in *RNR3* transcriptional repression, then treatment with the replication inhibitor, hydroxyurea (HU), is expected to induce expression of this gene in both a wild type and *asf1*Δ strain. Results from Northern blotting analysis revealed that *RNR3* is induced in both wild type and *asf1*Δ cells during S phase-arrest in response to HU (Fig. 3.4, A, B). This result is consistent with a normal DNA damage checkpoint being functional in the absence of Asf1, but does not rule out the possibility that the repressive effects exerted on *RNR3* by Asf1 are indirect. Evidence supporting a specific role for Asf1 in *RNR3* transcriptional regulation is provided by the microarray data. With the exception of the subset of genes in Figure 3.3, A, global transcriptional profiling for *asf1Δ* did not reveal significant changes in the majority of genes that are normally changed in expression level during the response to DNA damaging agents (Appendix Tables 7.1 and 7.4; Jelinsky and Samson, 1999). Finally, many genes involved in DNA repair pathways which include homologous recombination (HR) and nonhomologous end joining (NHEJ) were also not affected in the *asf1Δ* cells (Appendix Tables 7.1 and 7.4). In summary, induction of *RNR3* in *asf1*Δ cells is not due to misexpression of known regulators of *RNR3*, activation of the DNA damage checkpoint previously shown to impinge on *RNR3* (Huang et al., 1998), or abnormal cell cycle progression. When taken together with evidence that the DNA damage response pathway appears to be normal in *asf1*Δ cells, these findings are consistent with a specific role for Asf1 in *RNR3* transcriptional regulation that is independent of weak activation of the DNA damage checkpoint as it occurs in *asf1Δ* cells.

**In vivo association of Asf1 with *RNR3***

Based on the results of immunohistochemical, genetic, and biochemical studies, others have speculated that specific physical association of Asf1 with some genes might be a critical step in their transcriptional regulation by Asf1. Previous attempts using chromatin immunoprecipitation (ChIP), however, failed to reveal association of Asf1 with inducible genes which are misregulated in the absence of Asf1 (Adkins et al., 2004; Zabaronick and Tyler, 2005). Nonetheless, we used this methodology to test if Asf1 is normally present at the promoter and coding region of *RNR3* in vivo (Fig. 3.5, A shows PCR primer locations; techniques are outlined in Hecht and Grunstein, 1999). For quantitation we normalized the specific signal for each amplicon to recovery in the starting cell lysate ('Input'). This approach is widely used in the literature and is particularly appropriate for analysis of a histone chaperone which could potentially crosslink to DNA throughout the genome in the course of chromatin assembly coupled to replication (i.e. a priori, it is not possible to select a negative control region for normalization). HA-tagged Asf1 (Asf1-4HA; Meijsing and Ehrenhofer-Murray, 2001) expressed from a fusion construct ORF under control of the *ASF1* promoter was in fact readily detected across *RNR3* (Fig. 3.5, B). Asf1-4HA was also recovered in association with the promoters of *RNR2*, *RNR4*, *CRT1* (Fig. 3.6, A) and three tested histone genes whose cell cycle regulation may involve Asf1 (Sutton et al., 2001; Zabaronick and Tyler, 2004; Fig. 3.6, B). Control experiments using a strain expressing untagged Asf1 revealed that the ChIP signal is dependent on specific immunoprecipitation of Asf1-4HA (Fig. 3.6, A, B).

Because Asf1 is present in the coding region far downstream of the *RNR3* promoter, Asf1 recruitment to this locus may not exclusively involve its interaction with TFIID. This possibility is supported by our finding that Asf1 is present at the promoters of *ADH1* and *SED1* (Fig. 3.6, C), two well-characterized $TAF_{II}$-independent genes (Shen et al., 2003).

The results for *RNR3* do not reflect non-specific (genome-wide) association of Asf1 with chromatin because Asf1-4HA could not be crosslinked to the *FIS1* gene immediately upstream of *RNR3* (Fig. 3.3, B) or the promoter of *CUP1* or *PGK1* (Fig. 3.6, A, C). The negative results for Asf1 are also unlikely due to refractory behavior of the

target DNAs in PCR amplifications. For example, two other chromatin proteins, histone H3 and histone H2A variant Htz1, are readily detected in association with *FIS1* by ChIP (Fig. 3.7, C; see Chapter 4).

The results obtained using HA-tagged Asf1 and anti-HA antibody were confirmed when ChIP analysis was repeated using a strain expressing TAP-tagged Asf1 in place of the wild type protein (from the collection described in Ghaemmaghami et al., 2003). The strain expressing Asf1-TAP was not sensitive to MMS when compared to its wild type partner (J. Williams and M. Schultz, unpublished data). Therefore, the TAP tag does not appear to interfere with Asf1 function, as a *asf1Δ* cells are MMS-sensitive (Tyler et al., 1999; see also Chapter 4). In this ChIP experiment (Fig. 3.5, C), Asf1-TAP was recovered from lysate by incubation with rabbit IgG agarose. The level of non-specific binding of chromatin to the beads used for immunoprecipitation was determined by ChIP analysis of lysate from the isogenic wild type strain in which Asf1 is untagged. Crosslinking was monitored using primer pairs PR1, PR2, CD1 and CD2, because robust association of Asf1 with three of these regions of *RNR3* was observed in experiments using the Asf1-4HA-expressing strain (Fig. 3.5, B). Very little *RNR3* PCR product was generated when DNA in the immunoprecipitate from the untagged strain was amplified. On the other hand, a robust PCR signal was obtained for each probe after immunoprecipitation from the Asf1-TAP strain (Fig. 3.5, C). Based on the ChIP results for two different tagged versions of Asf1 we conclude that this protein is localized to the *RNR3* gene of yeast. To our knowledge this is the first demonstration of specific enrichment of Asf1 at any functional locus in a eukaryotic genome.

**Asf1 functions as a chromatin assembly/stabilization factor at *RNR3***

Taken together the evidence that Asf1 is a H3/H4 binding protein and our finding that Asf1 crosslinks to *RNR3* promoter and coding region DNA raises the possibility that Asf1 modulates the chromatin structure of *RNR3*. Two opposite molecular mechanisms could account for regulation of *RNR3* chromatin structure by Asf1. A large body of work suggests that Asf1 is a chromatin assembly factor. Perhaps then Asf1 contributes to nucleosome assembly at *RNR3*. On the other hand, Tyler and colleagues have recently presented data in favor of the view that Asf1 is a chromatin disassembly factor (Adkins et al., 2004; Adkins and Tyler, 2004). According to this model, localization of Asf1 to

*RNR3* would promote nucleosome disassembly at this locus. These divergent ideas about how Asf1 functions in chromatin metabolism lead to opposite predictions about how the chromatin organization of *RNR3* will be affected by *ASF1* deletion. If Asf1 functions as a chromatin assembly factor, then deletion of *ASF1* should be associated with disruption of chromatin structure at *RNR3* and (perhaps) reduced crosslinking of core histones to this locus. On the other hand, if Asf1 functions as a nucleosome disassembly factor, then its deletion should promote a more well ordered chromatin structure at *RNR3* and increased crosslinking of histones.

To distinguish between these possibilities we investigated how deletion of *ASF1* affects the chromatin configuration of *RNR3* and its crosslinking to a core histone. The chromatin organization of *RNR3* was analyzed by micrococcal nuclease digestion analysis of isolated nuclei followed by indirect end-labeling according to methods established by J. Reese and colleagues (Li and Reese, 2001). A representative experiment is presented in Figure 3.7, A. The pattern of bands resulting from digestion of wild type chromatin includes the expected hypersensitive sites which define the boundaries of positioned nucleosomes in the promoter and coding region of *RNR3* (lanes 2-4, filled circles; nucleosome numbering as in Li and Reese, 2001). Bands corresponding to these hypersensitive sites persist at all points in the micrococcal nuclease titration. The same bands are generated by digestion of chromatin from *asf1Δ* cells (lanes 6, 7). In the promoter, however, these bands (filled circles) are noticeably less well-defined than they are in digests of wild type chromatin, partly because of a more prominent background smear in the *asf1Δ* samples. This pattern may result from loss of nucleosomes or an increase in nucleosome mobility. *RNR3* promoter DNA is also much more susceptible to micrococcal nuclease digestion in *asf1Δ* than in wild type chromatin. Most notably, at the highest concentration of micrococcal nuclease used in Figure 3.7, A, none of the hypersensitive sites observed after digestion of wild type chromatin persists in the digests of *asf1Δ* chromatin (filled circles, compare lanes 4 and 8), and adjacent sites are also more sensitive to digestion. In addition to these differences in the chromatin structure of *RNR3* in wild type and *asf1Δ* cells, we also observe a faint hypersensitive site in the promoter of wild type chromatin that is not evident after digestion of *asf1Δ* chromatin (arrowhead), and a weak hypersensitive site in the coding

region of *asf1Δ* chromatin (open circle) that is not evident in wild type. It is important to note that the increased susceptibility of *RNR3* chromatin in *asf1Δ* cells to digestion is not a general property of the chromatin in that strain. For example, hypersensitive sites that define the boundaries of nucleosomes +2 and +3 in the coding region of *RNR3* are prominent in all digests of wild type and mutant chromatin. Furthermore, bulk chromatin in *asf1Δ* cells is not more susceptible to micrococcal nuclease digestion than chromatin in wild type cells (Fig. 3.7, B; see also Adkins and Tyler, 2004; Prado et al., 2004), and under normal growth conditions *ASF1* deletion is not associated with disruption of chromatin structure specifically at *PHO5* (Adkins et al., 2004). Collectively, the alterations in the pattern of micrococcal nuclease digestion associated with deletion of *ASF1* are consistent with specific disorganization of chromatin structure at *RNR3* rather than more robust assembly of chromatin. In other words, the results suggest that Asf1 contributes to the organization of chromatin at *RNR3* rather than its disorganization.

Association of histone H3 with *RNR3* in wild type and *asf1Δ* cells was examined by ChIP. We focused on regions of *RNR3* where Asf1 is highly enriched, namely the promoter and first two-thirds of the ORF. H3 was selected for this analysis because Asf1 is known to bind the H3/H4 tetramer, but not H2A or H2B. Furthermore, deletion of *ASF1* does not affect the bulk expression of H3 in the strain background used in this study (Fig. 3.2). For ChIP analysis, we used an antibody that recognizes the conserved C-terminus of H3 and has been extensively validated in the yeast literature (Adkins et al., 2004; Reinke and Horz, 2003). Two parallel control experiments were performed. The first revealed that background crosslinking of H3 to *RNR3* in mock immunoprecipitations is very low and not affected by deletion of *ASF1* (Fig 3.7, C, 'No antibody'). The second addressed the effect of *ASF1* deletion on H3 crosslinking to a region of chromosome V which is devoid of genes. For this experiment chromosome V primers were included in reactions which were also probed using *RNR3*-specific primers. As shown in the 'Chromosome V' panel of Figure 3.7, C, H3 crosslinking to this region of the genome was not significantly different between wild type and *asf1Δ* cells. Deletion of *ASF1* also does not affect H3 crosslinking to *FIS1* or the coding region of *RNR3*; it was essentially identical in four independent experiments, with little variability between replicates (mean ± SE for CD1 and CD2 primer sets is 1.01 ± 0.04 and 1.05 ± 0.03

respectively; Fig. 3.7, C). On the other hand, H3 crosslinking to the promoter of *RNR3* was consistently lower in the mutant than the wild type strain and H3 recovery varied substantially between replicate experiments (0.65 ± 0.12 for primer set PR2). In *asf1*Δ cells, reduced crosslinking of H3 at the promoter was statistically significant (P = 0.055 and 0.042 for comparison of H3 association with CD1 and CD2 respectively; Student's t-test, unequal variances, n = 4). Therefore expression of Asf1 is required for normal association of H3 with the promoter of *RNR3* but not its coding region. Reduced crosslinking of histones to *RNR3* in the absence of Asf1 is predicted by the model in which Asf1 functions as a chromatin assembly factor but not the model in which Asf1 functions as a disassembly factor. This reduced crosslinking is correlated with disruption of the chromatin organization of *RNR3* (above). We conclude that Asf1 functions at *RNR3* as a chromatin assembly and/or stabilization factor. It could be that Asf1 acts as a repressor by blocking other factors from association with *RNR3*. Because deletion of *ASF1* is associated with derepression of *RNR3*, we propose that promoter-targeted chromatin assembly/stabilization by Asf1 functionally contributes to repression of *RNR3* under normal conditions.

**DNA damage regulation of *RNR3* chromatin structure and transcription in *asf1*Δ cells**

We further tested if Asf1 is also required for normal chromatin reconfiguration and transcriptional induction of *RNR3* under conditions of genotoxic stress. The DNA damage response was elicited by treating wild type and *asf1*Δ cells with the alkylating agent methyl methanesulfonate (MMS), and remodeling was monitored by indirect end labeling after micrococcal nuclease digestion of nuclear chromatin. Two independent experiments yielded similar results. The results of one such experiment, presented in Figure 3.8, A, reveal that chromatin from MMS-treated *asf1Δ* cells (lanes 5-8) does not have the same susceptibility to micrococcal nuclease digestion as chromatin from similarly-treated wild type cells (lanes 1-4). Most notably, the intensity of hypersensitive sites flanking nucleosomes in the open reading frame (open circles) and immediately adjacent region of the promoter (closed circles) is reduced in the mutant. This difference is consistent with less well-organized *RNR3* chromatin in *asf1Δ* compared to wild type cells under conditions of genotoxic stress. Clearly deletion of *ASF1* affects both the

ground state of chromatin organization at *RNR3* (Fig. 3.7, A) and the outcome of remodeling triggered by genotoxic stress signals (Fig. 3.8, A).

Northern blotting was used to test if Asf1 also plays a role in the DNA damage regulation of *RNR3* transcription. Figure 3.8, B is a representative example of three independent experiments in which normalization to *ACT1* mRNA recovery was used to quantitate *RNR3* expression. Two effects of *ASF1* deletion on *RNR3* mRNA expression in MMS-treated cells were evident. First, *RNR3* is hyperinduced by MMS in cells that do not express Asf1. Similar hyperinduction has been reported for *isw2*Δ cells in which the chromatin structure of *RNR3* is also perturbed (Zhang and Reese, 2004a, b). Hyperinduction could be an indirect consequence of slowed progression through $G_1$, when *RNR3* is normally induced in some strain backgrounds (Cho et al., 1998; Spellman et al., 1998). The results of flow cytometry analysis of cells treated with MMS however argue against this possibility. Specifically, because a similar proportion of cells in wild type and *asf1*Δ cultures shifts to the $G_1$/S population upon exposure to MMS, elevated *RNR3* transcription in treated *asf1*Δ cultures is not likely due to accumulation of cells in $G_1$ (Fig. 3.8, C). The second effect of *ASF1* deletion on DNA damage regulation of *RNR3* is to reduce its fold stimulation in the presence of MMS. In Figure 3.8, B, *RNR3* induction due to MMS treatment is 2.1 fold lower in *asf1*Δ than wild type cells. Collectively our results establish that expression of Asf1 is required for normal chromatin remodeling and transcriptional induction of *RNR3* in response to DNA damage.

**Association of Asf1 with *RNR3* is regulated by DNA damage signals**

The effects of *ASF1* deletion on *RNR3* transcription generally resemble the previously reported effects of deleting *CRT1*, encoding the repressor which specifically binds to X boxes in the promoter of *RNR3* (see Table 1 in Huang et al., 1998). First, *RNR3* is derepressed under normal conditions in *crt1*Δ cells. Second, *RNR3* is hyperinduced when *crt1*Δ cells are treated with MMS. Finally, the fold stimulation of *RNR3* by DNA damage signals is substantially dampened in *crt1*Δ compared to wild type cells. Under conditions of genotoxic stress repression of *RNR3* by Crt1 is relieved because Crt1 dissociates from the promoter (Huang et al., 1998). The overall similarity of the effect of *ASF1* and *CRT1* deletion on *RNR3* expression, and the fact that both Asf1 and Crt1 locate to the promoter under normal conditions, led us to test if DNA damage

signals also trigger dissociation of Asf1 from *RNR3*. The occupancy of *RNR3* by HA-tagged Asf1 before and after treatment with MMS was determined by ChIP. As shown in Figure 3.9, A, there was a dramatic loss of Asf1-4HA from the promoter and coding region of *RNR3* in response to genotoxic stress. Global degradation of Asf1 does not account for this effect since bulk expression of previously described Asf1 species (Emili et al., 2001) is not sensitive to MMS treatment (Fig. 3.9, B). Neither is reduced crosslinking of Asf1 to *RNR3* a reflection of pervasive disruption of protein-DNA interactions at *RNR3*. For example, MMS treatment does not affect crosslinking of histones to the coding region (Zhang and Reese, 2004a; J. Williams and M. Schultz, unpublished data), from which Asf1 completely dissociates (Fig. 3.9, A). Finally, this regulation does not universally affect chromatin-associated Asf1, since Asf1 crosslinking to some other gene promoters is either unaffected or declines only modestly upon exposure to MMS (Fig. 3.10).

Overall our results suggest that dynamic localization of Asf1 to *RNR3* is an important step in the normal regulation of this gene. Specifically we propose that repression by promoter nucleosomes is relieved under conditions of genotoxic stress partly because nucleosome assembly is disrupted, or nucleosomes are destabilized, when Asf1 is dissociated. This model is strongly supported by the recent observation that H4 crosslinking to the promoter of *RNR3* is significantly reduced when cells are treated with MMS (Zhang and Reese, 2004a).

**Discussion:**

We provide the first direct evidence that dynamic gene targeting of conserved nucleosome assembly factor Asf1 is an important step in chromatin remodeling associated with transcriptional reprogramming.

**Distinct transcriptional roles for Asf1 and CAF-1**

Histone chaperones Asf1 and CAF-1 are involved in replication-dependent and replication-independent chromatin assembly (Smith and Stillman, 1989; Kaufman et al., 1995; Tyler et al., 1999; Munakata et al., 2000; Robinson and Schultz, 2003). Microarray analysis revealed that individual deletion of *ASF1* or subunits of CAF-1 resulted in similar yet distinct transcriptional profiles. This may reflect the different roles that these factors have during chromatin assembly, as well as other processes such as transcriptional silencing and DNA repair. A current model for Asf1 function during replication-dependent chromatin assembly, in which Asf1 delivers histones to DNA directly or in a CAF-1 dependent manner, may help explain these differences and the overall increased transcriptional consequence of *ASF1* deletion when compared to either *cac1Δ* or *msi1Δ* cells (Table 3.2; Mello and Almouzni, 2001). Furthermore, deletion of *ASF1* results in a more severe silencing and DNA damage-sensitivity phenotype than deletion of CAF-1 subunits (Enomoto et al., 1997; Kaufman et al., 1997; Tyler et al., 1999). Regardless, it is important to note that while our microarray analysis has identified those transcripts that are affected by deletion of these CAFs, many of these effects may be indirect.

**Histone chaperone targeting and chromatin-dependent repression of *RNR3***

Asf1 was originally identified as a factor that disrupts transcriptional silencing at yeast telomeres when overexpressed (Le et al., 1997; Singer et al., 1998). The subsequent discovery that Asf1 is required for transcriptional regulation of histone genes led to speculation that it might be targeted to some non-telomeric loci and function in transcription at such loci by affecting chromatin organization (Sutton et al., 2001). The results of our study confirm these general predictions and support a new model in which specific, reversible association of Asf1 with a target gene modulates its nucleosome-dependent repression.

The straightforward interpretation of our results is that Asf1 targeted to *RNR3* represses transcription by contributing to nucleosome assembly specifically in the

promoter of this gene. In support of this conclusion we find that Asf1 is required under benign conditions for normal crosslinking of H3 to the promoter of *RNR3*, normal organization of its chromatin, and full repression of transcription. This proposed mechanism of action is consistent with an extensive literature showing that soluble Asf1 can bind to histones H3 and H4 and transfer them to DNA in a configuration favorable for nucleosome formation (reviewed in Haushalter and Kadonaga, 2003). No histone chaperone identified to date is essential for viability, and nucleosomes can be reconstituted from purified histones and DNA by salt gradient dialysis in vitro. However, under physiological ionic strength conditions, chromatin assembly factors such as Asf1 are required to overcome the nonspecific association between histones and DNA that results in insoluble aggregates (Verreault, 2000). It is important to note that no chromatin assembly factor identified to date in *S. cerevisiae* is essential, highlighting their functional redundancy and the possibility that other chromatin-associated proteins regulate chromatin assembly as well.

It is also possible that *RNR3*-associated Asf1 binds to H3/H4 in promoter nucleosomes and protects the nucleosomes from destabilization by trans-acting chromatin remodeling factors, although there is no direct biochemical evidence to support this idea. Because of the specialized and dynamic roles for chromatin in regulation of gene expression and cell growth, the chromatin structure established and/or maintained by Asf1 may be central to this regulation. It is interesting to speculate that formation of a specialized chromatin structure at Asf1 target genes may further involve the previously identified interactions of Asf1 with chromatin remodeling enzymes such as SWI/SNF (Moshkin et al., 2002) and histone modifying enzymes such as the SAS histone acetyltransferase complex (Meijsing and Ehrenhofer-Murray, 2001; Osada et al., 2001).

Recent in vivo analysis of the *PHO5* and *PHO8* genes of yeast suggests that Asf1 can function in chromatin metabolism by removing H3/H4 from the DNA (Adkins et al., 2004). This activity of Asf1 likely facilitates transcription by disassembling inhibitory nucleosomes. Regulation by this mechanism (as it is currently understood) is excluded at *RNR3* for the following reasons. First, in the nucleosome disassembly model transcriptional induction is expected to fail in *asf1*Δ cells (assuming induction at this gene requires nucleosome disassembly). We find the reverse: *RNR3* is hyper-induced in *asf1*Δ

cells. Second, the disassembly model predicts more robust association of H3 with DNA in *asf1*Δ than wild type cells. The reverse is true at *RNR3*: H3 is less robustly associated with promoter DNA in *asf1*Δ than wild type cells (as judged by ChIP analysis). Third, Adkins et al. (2004) predict that nucleosome disassembly by Asf1 will involve its specific association with target genes under inducing conditions. Again we find the reverse: Asf1 crosslinking to *RNR3* is very weak under inducing conditions and very robust under repressing conditions. While Asf1 does not disassemble nucleosomes at *RNR3*, its ability to facilitate transcription by both targeted nucleosome assembly (this study) and nucleosome disassembly (Adkins and Tyler, 2004; Adkins et al., 2004) likely accounts for the observed repression as well as induction of transcription in *asf1*Δ cells (Fig. 3.1; Appendix Tables 7.1 and 7.4), and the fact that in wild type cells Asf1 is normally localized to some genes that are induced and others that are repressed when Asf1 is not expressed (Table 3.10).

Why Asf1 is present in the coding region of *RNR3* remains unknown. Its regulation by DNA damage signals suggests a role in transcription. One possibility is that Asf1 binds to and protects previously assembled nucleosomes from spurious remodeling not involving histone dissociation. This is a plausible idea given that another histone chaperone, Nap1, can assemble nucleosomes, facilitate their disassembly, and potently inhibit histone acetylation (Asahara et al., 2002; Ito et al., 2000).

**DNA damage regulation of Asf1 molecules involved in targeted repression of *RNR3***

DNA damage signals presumably over-ride the mechanisms that govern association of Asf1 with *RNR3* under normal conditions. Our working model for DNA damage regulation of Asf1 at *RNR3* is based on what is known about DNA damage regulation of the soluble complex that includes Asf1 and Rad53. When cells experience genotoxic stress, this complex is disrupted by a still unknown mechanism so as to release Asf1 (Emili et al., 2001; Hu et al., 2001). By extension, we propose that molecular interactions of Asf1 that contribute to its association with *RNR3* under benign conditions are disrupted by DNA damage signals, which in turn leads to wholesale dissociation of Asf1 from the *RNR3* locus (Fig. 3.9, A). The repressive effects of Asf1 on transcription are then relieved. Our analysis of H3 association with *RNR3* in *asf1*Δ cells suggests how regulation of Asf1 contributes to induction of *RNR3* by DNA damage signals.

Specifically we envisage that repression is dampened at the promoter when cells experience genotoxic stress because dissociation of Asf1 inhibits H3/H4 deposition into nucleosomes (Fig. 3.11). This model is strongly supported by evidence that crosslinking of H4 over the promoter of *RNR3* declines significantly in cells treated with MMS (Zhang and Reese, 2004a). Because H3 association with coding region DNA is unaffected by deletion of *ASF1* (Fig. 3.7, C), H3/H4 tetramer dissociation from the coding region is not expected to accompany loss of Asf1 under conditions of genotoxic stress (Fig. 3.9, A). That indeed is the case; H4 crosslinking over the coding region of *RNR3* is largely unaffected by MMS treatment (Zhang and Reese, 2004a).

DNA damage signals lead to Rad53 activation and subsequent induction of *RNR3* transcription (Fig. 3.3, C; Fig. 3.8, B; Sanchez et al., 1996; Sun et al., 1996; Huang et al., 1998). Our evidence indicates that the possible weak activation of Rad53 as it occurs in *asf1Δ* cells does not lead to *RNR3* induction. This could be due to the threshold effects associated with Rad53 activation. Shimada et al. (2002) proposed that a certain threshold number of stalled replication forks during S phase are required to activate the DNA damage checkpoint. It may be that full activation of Rad53 by phosphorylation at multiple sites is required for transcriptional activation of DNA damage response genes such as *RNR3*. Furthermore, loss of the Arp8 subunit of the INO80 chromatin remodeling complex results in a similar shift in Rad53 mobility as that observed in cycling *asf1Δ* cells (Fig. 3.3, C; van Attikum et al., 2004). However, global expression analysis revealed similar *RNR3* expression levels in *arp8Δ* and wild type strains. Therefore, it appears that a certain threshold of Rad53 activation is required for initiation of the DNA damage response and transcriptional induction of *RNR3*. Our results are consistent with a model in which this threshold is not reached in *asf1Δ* cells, and consequently the possible weak activation of Rad53 in *asf1Δ* cells is not responsible for *RNR3* transcriptional induction.

The existing evidence reveals that DNA damage regulation of Asf1 contributes to chromatin metabolism in two distinct biochemical pathways. Specifically, genotoxic stress signals both activate global, DNA repair-coupled assembly of nucleosome arrays by soluble Asf1 (Emili et al., 2001; Mello et al., 2002), and inhibit functions of *RNR3*-localized Asf1 which promote the repressed state of chromatin at *RNR3* (this study).

Being the target of DNA damage signals in both these contexts, Asf1 is in a unique position to coordinate fundamentally dissimilar chromatin remodeling events which are important for cell survival in the face of genotoxic stress. It will be interesting to test in the future if other histone chaperones have divergent roles which are regulated by the same physiological signal so as to coordinate steps in chromatin metabolism that have functionally distinct outcomes.

**Table 3.1.** Yeast strains used in Chapter 3

| Strain | Genotype |
|---|---|
| BY4741[a] | MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| BY4741 asf1Δ[a] | MATa *asf1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| BY4741 cac1Δ[a] | MATa *cac1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| BY4741 msi1Δ[a] | MATa *msi1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| YJW1 | BY4741 [pYEX-GST-ASF1][b] |
| YJW3 | BY4741 [pYEX-GST][b] |
| YJW6[c] | MATa *leu2 trp1 ura3-52 prc1-407 pep4-3 prb1-112 ASF1-4HA::TRP1* |
| YJW7[d] | MATa *ade2-1 ura3-1 his3-11,15 leu2-3, 112 trp1-1* |
| YJW91 | BY4741 *asf1Δ* [pYEX-GST][b] |
| YJW94 | BY4741 *asf1Δ* [pYEX-GST-ASF1][b] |

[a]Supplied by Open Biosystems.
[b]Plasmids from the collection of Martzen et al., 1999 (supplied by Research Genetics). Strains transformed with pYEX-GST-ASF1 expressed the correctly sized GST fusion protein and the plasmid fully complemented the growth and MMS-sensitivity phenotypes of *asf1Δ* cells.
[c]Strain from Ann Ehrenhofer-Murray (Meijsing and Ehrenhofer-Murray, 2001).
[d]Strain from Rodney Rothstein (W303-1a; Thomas and Rothstein, 1989).

**Table 3.2.** Summary of microarray results for *asf1Δ*, *cac1Δ*, and *msi1Δ* cells

| Strain | Average # genes upregulated > 2 fold (% of genome) | Average # genes downregulated > 2 fold (% of genome) |
|---|---|---|
| *asf1Δ* | 624 (9.8%) | 121 (1.9%) |
| *cac1Δ* | 211 (3.3%) | 101 (1.6%) |
| *msi1Δ* | 146 (2.3%) | 98 (1.5%) |

**Table 3.3.** Genes involved in cellular metabolism that are upregulated in *asf1Δ* cells

| Gene | Average Fold Δ | ORF and Description |
|---|---|---|
| | 38 | YGR052W similarity to ser/thr protein kinases |
| *CRC1* | 34 | YOR100C similarity to mitochondrial carrier proteins |
| *BNA2* | 32 | YJR078W sim. to mammalian indoleamine 2,3-dioxygenase |
| | 23 | YJL045W similarity to succinate dehydrogenase flavoprotein |
| | 20 | YPL230W Up in Starvation |
| *DAL1* | 16.6 | YIR027C allantoinase |
| *INH1* | 13 | YDL181W ATPase inhibitor |
| *GPH1* | 12.6 | YPR160W Glycogen phosphorylase |
| *CYC7* | 9.5 | YEL039C iso-2-cytochrome c |
| *GSP2* | 7.7 | YOR185C GTP binding protein, almost identical to Gsp1p |
| *SGA1* | 7.7 | YIL099W intracellular glucoamylase |
| | 7 | YMR118C strong similarity to succinate dehydrogenase |
| *HXT4* | 6.7 | YHR092C High-affinity glucose transporter |
| *INO1* | 6.5 | YJL153C L-myo-inositol-1-phosphate synthase |
| *YGP1* | 6.3 | YNL160W YGP1 encodes gp37, a glycoprotein |
| | 6.1 | YGR043C strong similarity to transaldolase |
| *ARG1* | 5.9 | YOL058W arginosuccinate synthetase |
| *NCA3* | 5.9 | YJL116C regulates expression Fo-F1 ATP synthase subunits |
| *JEN1* | 5.9 | YKL217W carboxylic acid transporter protein homolog |
| *FBP1* | 5.7 | YLR377C fructose-1,6-bisphosphatase |
| *HOR2* | 5.7 | YER062C DL-glycerol-3-phosphatase |
| | 5.7 | YFL030W similarity to several transaminases |
| *DIT1* | 5.3 | YDR403W first enzyme in dityrosine synthesis |
| *YPS5* | 5.3 | YGL259W GPI-anchored aspartic protease |
| | 5.3 | YOL155C similarity to glucan 1,4-alpha-glucosidase |
| *PGM2* | 5.1 | YMR105C Phosphoglucomutase |
| *HXK1* | 5.1 | YFR053C Hexokinase I (PI) (also called Hexokinase A) |
| *COX9* | 4.9 | YDL067C Subunit VIIa of cytochrome c oxidase |
| *IDP1* | 4.9 | YDL066W Mito. form of NADP isocitrate dehydrogenase |
| | 4.9 | YOL155C similarity to glucan 1,4-alpha-glucosidase Sta1p |
| *HXT9* | 4.9 | YJL219W High-affinity hexose transporter |
| *SPS19* | 4.9 | YNL202W peroxisomal 2,4-dienoyl-CoA reductase |
| *GAP1* | 4.8 | YKR039W general amino acid permease |
| *CYC1* | 4.8 | YJR048W iso-1-cytochrome c |
| *STF1* | 4.8 | YDL130W-A ATPase stabilizing factor |
| *YPS6* | 4.8 | YIR039C GPI-anchored aspartic protease |
| *GAD1* | 4.4 | YMR250W similarity to glutamate decarboxylases |

| | | |
|---|---|---|
| *COX4* | 4.4 | YGL187C subunit IV of cytochrome c oxidase |
| *TKL2* | 4.4 | YBR117C transketolase, homologous to tkl1 |
| *ARG3* | 4.3 | YJL088W Ornithine carbamoyltransferase |
| *CTT1* | 4.3 | YGR088W cytoplasmic catalase T |
| | 4 | YLR004C similarity to allantoate transport protein |
| *GLC3* | 4 | YEL011W 1,4-glucan-6-(1,4-glucano)-transferase |
| *ARG8* | 3.9 | YOL140W Acetylornithine aminotransferase |
| *MLS1* | 3.9 | YNL117W carbon-catabolite sensitive malate synthase |
| *PUT4* | 3.9 | YOR348C putative proline-specific permease |
| *ATP2* | 3.9 | YJR121W F(1)F(0)-ATPase complex beta subunit |
| *ALD3* | 3.9 | YMR169C Aldehyde Dehydrogenase (NAD(P)+) |
| *UGA2* | 3.7 | YBR006W Probable aldehyde dehydrogenase |
| *QCR2* | 3.7 | YPR191W ubiquinol cytochrome-c reductase core protein 2 |
| *MEP2* | 3.7 | YNL142W Ammonia transport protein |
| | 3.6 | YPL017C similarity to dihydrolipoamide dehydrogenases |
| *AUT4* | 3.6 | YCL038C Membrane transporter |
| *ADH2* | 3.6 | YMR303C alcohol dehydrogenase II |
| *GDB1* | 3.5 | YPR184W similarity to human 4-alpha-glucanotransferase |
| | 3.5 | YAL061W similarity to alcohol/sorbitol dehydrogenase |
| *HXT5* | 3.5 | YHR096C hexose transporter |
| *CPS1* | 3.5 | YJL172W carboxypeptidase yscS |
| *MAL11* | 3.5 | YGR289C alpha-glucoside transporter |
| | 3.4 | YNR073C similarity to E.coli D-mannonate oxidoreductase |
| *FCY22* | 3.4 | YER060w-A purine-cytosine permease |
| *CIT3* | 3.4 | YPR001W Mitochondrial isoform of citrate synthase |
| | 3.2 | YFL057C strong similarity to aryl-alcohol dehydrogenases |
| *GSY2* | 3.2 | YLR258W Glycogen synthase |
| *QCR10* | 3.1 | YHR001W ubiqunol-cyt. c oxidoreductase complex subunit |
| *THI4* | 3.1 | YGR144W thiamine biosynthetic pathway component |
| *VPS73* | 3.1 | YGL104C similarity to glucose transport proteins |
| *ODC1* | 3.1 | YPL134C similarity to ADP/ATP carrier proteins |
| *GPM2* | 3.1 | YDL021W Similar to GPM1 (phosphoglycerate mutase) |
| *COX5A* | 3 | YNL052W Cytochrome-c oxidase chain Va |
| *RPM2* | 3 | YML091C subunit of mitochondrial RNase P |
| *MCR1* | 3 | YKL150W NADH-cytochrome b5 reductase |
| | 3 | YKL161C probable serine\threonine-specific protein kinase |
| *LSC2* | 3 | YGR244C Succinate-CoA Ligase (ADP-Forming) |
| *URA10* | 3 | YMR271C Orotate phosphoribosyltransferase 2 |
| *BNA1* | 3 | YJR025C 3-hydroxyanthranilic acid dioxygenase |
| *HXT6* | 3 | YDR343C Hexose transporter |
| *LAP4* | 3 | YKL103C vacuolar aminopeptidase ysc1 |
| *DAL4* | 3 | YIR028W allantoin permease |

**Table 3.4.** Genes involved in cellular metabolism that are upregulated in *cac1Δ* cells

| Gene | Average Fold Δ | ORF and Description |
|---|---|---|
| | 55.7 | YGR111W weak similarity to mosquito carboxylesterase |
| | 26.9 | YJR078W similarity to indoleamine 2,3-dioxygenase |
| | 21.9 | YNR064C similarity to 1-chloroalkane halidohydrolase |
| *SPO1* | 21.9 | YNL012W high similarity to phospholipase B |
| | 19 | YNL335W sim. to *M.verrucaria* cyanamide hydratase |
| *PPQ1* | 2.9 | YPL179W protein phosphatase Q |
| *THI11* | 13.9 | YJR156C Thiamine biosynthetic enzyme |
| *ADH2* | 10.9 | YMR303C alcohol dehydrogenase II |
| | 10.2 | YMR118C strong similarity to succinate dehydrogenase |
| *FDH1* | 9.8 | YOR388C similarity to formate dehydrogenases |
| *DAL1* | 9.8 | YIR027C allantoinase |
| | 8.3 | YLL057C similarity to E.coli dioxygenase |
| | 7.7 | YJL045W sim. to succinate dehydrogenase flavoprotein |
| *JEN1* | 7 | YKL217W carboxylic acid transporter protein homolog |
| *TKL2* | 6.5 | YBR117C transketolase, homologous to tkl1 |
| *HXT9* | 6.3 | YJL219W High-affinity hexose transporter |
| *SPS19* | 5.5 | YNL202W peroxisomal 2,4-dienoyl-CoA reductase |
| *GND2* | 4.1 | YGR256W 6-phosphogluconate dehydrogenase |
| *PGU1* | 4 | YJR153W Endo-polygalacturonase |
| *GPH1* | 4 | YPR160W Glycogen phosphorylase |
| *INO1* | 4 | YJL153C L-myo-inositol-1-phosphate synthase |
| *GAL7* | 3.9 | YBR018C galactose-1-phosphate uridyl transferase |
| *DIT1* | 3.9 | YDR403W first enzyme in dityrosine synthesis |
| *YPS5* | 3.9 | YGL259W GPI-anchored aspartic protease |
| | 3.7 | YNR062C similarity to *H. influenzae* L-lactate permease |
| *FBP1* | 3.7 | YLR377C fructose-1,6-bisphosphatase |
| *GAL10* | 3.6 | YBR019C UDP-glucose 4-epimerase |
| | 3.5 | YNR073C sim. to *E. coli* D-mannonate oxidoreductase |
| *MLS1* | 3.5 | YNL117W carbon-catabolite sensitive malate synthase |
| *CYB2* | 3.5 | YML054C Cytochrome b2 |
| *YPS6* | 3.2 | YIR039C GPI-anchored aspartic protease |
| | 3.2 | YPL113C similarity to glycerate dehydrogenases |
| *OYE3* | 3.1 | YPL171C NAD(P)H dehydrogenase |
| *PDC5* | 3.1 | YLR134W pyruvate decarboxylase |
| *BIO3* | 3 | YNR058W 7,8-diamino-pelargonic acid aminotransferase |
| *ZWF1* | 2.9 | YNL241C Glucose-6-phosphate dehydrogenase |
| *SDH2* | 2.9 | YLL041C Succinate dehydrogenase iron-sulfur protein |

| | | |
|---|---|---|
| *GDH3* | 2.9 | YAL062W NADP-linked glutamate dehydrogenase |
| *POT1* | 3 | YIL160C peroxisomal 3-oxoacyl CoA thiolase |
| *HXT16* | 2.8 | YJR158W hexose transporter |
| *GLG2* | 2.8 | YJL137C self-glucosylating initiator of glycogen synthesis |
| *PGM2* | 2.7 | YMR105C Phosphoglucomutase |
| *PHO5* | 2.7 | YBR093C Acid phosphatase, repressible |
| | 2.6 | YGR043C strong similarity to transaldolase |
| *GSY2* | 2.5 | YLR258W Glycogen synthase |
| *TES1* | 2.5 | YJR019C peroxisomal acyl-CoA thioesterase |
| *XYL2* | 2.5 | YLR070C strong similarity to sugar dehydrogenases |
| *MAL33* | 2.5 | YBR297W Maltose fermentation regulatory protein |
| | 2.5 | YFL030W similarity to several transaminases |
| *THI4* | 2.5 | YGR144W thiamine biosynthetic pathway component |
| *AAD4* | 2.5 | YDL243C Hypothetical aryl-alcohol dehydrogenase |
| *MAL32* | 2.4 | YBR299W Maltase (EC 3.2.1.20) |
| *GDH3* | 2.4 | YAL062W NADP-linked glutamate dehydrogenase |
| *FRM2* | 2.3 | YCL026C-A involved in the integration of lipid signaling |
| *CAT2* | 2.3 | YML042W Carnitine O-acetyltransferase |
| *DAL4* | 2.3 | YIR028W allantoin permease |
| *CYR1* | 2.2 | YJL005W adenylate cyclase |
| *PCK1* | 2.2 | YKR097W phosphoenolpyruvate carboxylkinase |
| *HXK1* | 2.2 | YFR053C Hexokinase I (PI) (also called Hexokinase A) |
| *DAL3* | 2.1 | YIR032C ureidoglycolate hydrolase |
| | 2.1 | YER119C similarity to *E. herbicola* tyrosine permease |
| *MEP2* | 2.1 | YNL142W Ammonia transport protein |
| | 2.1 | YOL132W similarity to surface glycoprotein Gas1p |
| | 2.1 | YLR004C similarity to allantoate transport protein |
| *SGA1* | 2.1 | YIL099W intracellular glucoamylase |
| | 2 | YDR516C strong similarity to glucokinase |

**Table 3.5.** Genes involved in cellular metabolism that are upregulated in *msi1Δ* cells

| Gene | Avgerage Fold Δ | ORF and Description |
|---|---|---|
| | 55.7 | YGR111W weak similarity to mosquito carboxylesterase |
| *SPO1* | 16.6 | YNL012W similarity to phospholipase B |
| | 14.4 | YNR064C similarity to *R. capsulatus* halidohydrolase |
| | 12.1 | YJR078W sim. to indoleamine 2,3-dioxygenase |
| | 6.7 | YMR118C strong similarity to succinate dehydrogenase |
| *THI11* | 6.5 | YJR156C Thiamine biosynthetic enzyme |
| *TKL2* | 5.9 | YBR117C transketolase, homologous to tkl1 |
| *FDH1* | 5.9 | YOR388C similarity to formate dehydrogenases |
| *ADH2* | 5.7 | YMR303C alcohol dehydrogenase II |
| *YPS6* | 4.4 | YIR039C GPI-anchored aspartic protease |
| | 4.1 | YNR062C similarity to *H. influenzae* L-lactate permease |
| *YPS5* | 4 | YGL259W GPI-anchored aspartic protease |
| *SPS19* | 3.9 | YNL202W peroxisomal 2,4-dienoyl-CoA reductase |
| *JEN1* | 3.9 | YKL217W carboxylic acid transporter protein homolog |
| *GND2* | 3.7 | YGR256W 6-phosphogluconate dehydrogenase |
| | 3.6 | YGL104C similarity to glucose transport proteins |
| *GPH1* | 3.4 | YPR160W Glycogen phosphorylase |
| | 3.4 | YER119C similarity to *E. herbicola* tyrosine permease |
| | 3.4 | YNR073C sim. to *E. coli* D-mannonate oxidoreductase |
| | 3.4 | YJL045W strong similarity to succinate dehydrogenase |
| *THI4* | 3.2 | YGR144W thiamine biosynthetic pathway component |
| *PHO5* | 3.1 | YBR093C Acid phosphatase, repressible |
| *GAL7* | 2.9 | YBR018C galactose-1-phosphate uridyl transferase |
| *GAL10* | 2.8 | YBR019C UDP-glucose 4-epimerase |
| *PDC5* | 2.8 | YLR134W pyruvate decarboxylase |
| *FCY22* | 2.8 | YER060w-A purine-cytosine permease |
| *DIT1* | 2.7 | YDR403W first enzyme in dityrosine synthesis |
| | 2.7 | YFL030W similarity to several transaminases |
| *SGA1* | 2.6 | YIL099W intracellular glucoamylase |
| *FBP1* | 2.6 | YLR377C fructose-1,6-bisphosphatase |
| *MEP2* | 2.5 | YNL142W Ammonia transport protein |
| *MAL33* | 2.5 | YBR297W Maltose fermentation regulatory protein |
| | 2.5 | YFR018C sim. to glutaminyl-peptide cyclotransferase |
| *PGU1* | 2.5 | YJR153W Endo-polygalacturonase |
| *ARE1* | 2.4 | YCR048W Acyl-CoA cholesterol acyltransferase |
| *RHK1* | 2.3 | YBL082C putative alpha(1-3) mannosyltransferase |
| *DUR3* | 2.3 | YHL016C Urea transporter |

| | | |
|---|---|---|
| *XYL2* | 2.3 | YLR070C strong similarity to sugar dehydrogenases |
| *BIO3* | 2.2 | YNR058W 7,8-diamino-pelargonic acid aminotransferase |
| *OYE3* | 2.2 | YPL171C NAD(P)H dehydrogenase |
| *HXT9* | 2.2 | YJL219W High-affinity hexose transporter |
| *CHS7* | 2.1 | YHR142W weak similarity to cytochrome-c oxidases |
| *GDH3* | 2 | YAL062W NADP-linked glutamate dehydrogenase |

**Table 3.6.** Genes encoding ribosomal proteins or Ty transposable elements that are downregulated in *asf1Δ* cells

| Gene | Average Fold Δ | ORF and Description |
|---|---|---|
| | -4.6 | YJLWDELTA19 Ty1 LTR |
| | -3.9 | YILCDELTA2 Ty2 LTR |
| | -3.4 | YERWDELTA18 Ty1 LTR |
| | -2.9 | YERWDELTA18 Ty1 LTR |
| *RPS23B* | -2.5 | YPR132W Ribosomal protein S23B (S28B) (rp37) (YS14) |
| *RPL24A* | -2.5 | YGL031C Ribosomal protein L24A (rp29) (YL21) (L30A) |
| *RDN18-1* | -2.2 | RDN18-1 18S ribosomal RNA |
| *RPL30* | -2.2 | YGL030W Large ribosomal subunit protein L30 (L32) |
| *RPL1A* | -2.2 | YPL220W Ribosomal protein L1A of the 60S subunit |
| *RPP0* | -2.2 | YLR340W 60S ribosomal protein P0 (A0) (L10E) |
| *RPL10* | -2.3 | YLR075W Ribosomal protein L10 |
| *RPP2B* | -2.3 | YDR382W Ribosomal protein P2B (YP2beta) (L45) |
| *RPL11A* | -2.3 | YPR102C Ribosomal protein L11A (L16A) (rp39A) (YL22) |
| *RPS31* | -2.3 | YLR167W Ribosomal protein S31 (S37) (YS24) |
| *RPL31A* | -2.2 | YDL075W Ribosomal protein L31A (L34A) (YL28) |
| *RPS9B* | -2.2 | YBR189W Ribosomal protein S9B (S13) (rp21) (YS11) |
| *RPS12* | -2.2 | YOR369C 40S ribosomal protein S12 |
| *RPL26A* | -2.2 | YLR344W Ribosomal protein L26A (L33A) (YL33) |
| *RPS21A* | -2.2 | YKR057W Ribosomal protein S21A (S26A) (YS25) |
| *RPS2* | -2.2 | YGL123W Ribosomal protein S2 (S4) (rp12) (YS5) |
| *RPL2A* | -2.2 | YFR031C-A Ribosomal protein L2A (L5A) (rp8) (YL6) |
| *RDN37-1* | -2.2 | RDN37-1 35S ribosomal RNA |
| *RPS10A* | -2.1 | YOR293W Ribosomal protein S10A |
| *RPL14A* | -2.1 | YKL006W Ribosomal protein L14A |
| *RPL4A* | -2.1 | YBR031W Ribosomal protein L4A (L2A) (rp2) (YL2) |
| *RPL20A* | -2.1 | YMR242C Ribosomal protein L20A (L18A) |
| *RPL16A* | -2.1 | YIL133C Ribosomal protein L16A (L21A) (rp22) (YL15) |
| *RPS9B* | -2.1 | YBR189W Ribosomal protein S9B (S13) (rp21) (YS11) |
| *RPS8A* | -2.1 | YBL072C Ribosomal protein S8A (S14A) (rp19) (YS9) |
| *RPS17A* | -2.1 | YML024W Ribosomal protein S17A (rp51A) |
| *RPL24B* | -2.1 | YGR148C Ribosomal protein L24B (rp29) (YL21) (L30B) |
| *RPL9A* | -2.1 | YGL147C Ribosomal protein L9A (L8A) (rp24) (YL11) |
| *RPS3* | -2.1 | YNL178W Ribosomal protein S3 (rp13) (YS3) |
| *RPL23A* | -2.1 | YBL087C Ribosomal protein L23A (L17aA) (YL32) |
| *RPS21B* | -2.1 | YJL136C Ribosomal protein S21B (S26B) (YS25) |
| | -2.1 | YERWDELTA18 Ty1 LTR |
| *RPL34B* | -2.1 | YIL052C Ribosomal protein L34B |
| *RPL11A* | -2.1 | YPR102C Ribosomal protein L11A (L16A) (rp39A) (YL22) |
| *RPS6A* | -2.1 | YPL090C Ribosomal protein S6A (S10A) (rp9) (YS4) |

| | | |
|---|---|---|
| *RPS10B* | -2.1 | YMR230W Ribosomal protein S10B |
| *RPS22A* | -2.1 | YJL190C Ribosomal protein S22A (S24A) (rp50) (YS22) |
| | -2.1 | YERWDELTA18 Ty1 LTR |
| *RPS27B* | -2.1 | YHR021C 40S Ribosomal protein S27B (rp61) (YS20) |
| *RPL28* | -2.1 | YGL103W Ribosomal protein L28 (L29) (rp44) (YL24) |
| *RPL4B* | -2.1 | YDR012W Ribosomal protein L4B (L2B) (rp2) (YL2) |
| *RPP1B* | -2.1 | YDL130W Ribosomal protein P1B (L44 ) (YP1beta) (Ax) |
| *RPL5* | -2.1 | YPL131W Ribosomal protein L5 (L1a)(YL3) |
| *RPL18B* | -2.1 | YNL301C Ribosomal protein L18B (rp28B) |
| *RPS7A* | -2 | YOR096W Ribosomal protein S7A (rp30) |
| *RPL14A* | -2 | YKL006W Ribosomal protein L14A |
| *RPL8B* | -2 | YLL045C Ribosomal protein L8B (L4B) (rp6) (YL5) |
| *RPS4A* | -2 | YJR145C Ribosomal protein S4A (YS6) (rp5) (S7A) |
| *RPL43A* | -2 | YPR043W Ribosomal protein L43A |
| *RPS1A* | -2 | YLR441C Ribosomal protein S1A (rp10A) |
| *RPL17B* | -2 | YJL177W Ribosomal protein L17B (L20B) (YL17) |

**Table 3.7.** Genes involved in RNA processing or encoding subunits of RNA polymerases I, II, or III that are downregulated in *cac1Δ* cells

| Gene | Average Fold Δ | ORF and Description |
|---|---|---|
| *FYV14* | -2.7 | YDL213C has an RNA recognition domain in the N-terminus |
| *RRP3* | -2.7 | YHR065C encodes an RNA-dependent ATPase (DEAD box) |
| *RRP7* | -2.7 | YCL031C involved in rRNA processing, ribosome assembly |
| *SRB7* | -2.6 | YDR308C RNA polymerase II holoenzyme component |
| *BDF1* | -2.6 | YLR399C contains two bromodomains |
| *IST3* | -2.5 | YGR029W essential for mitochondrial biogenesis, viability |
| *RRP1* | -2.5 | YDR087C involved in processing rRNA precursor species |
| *YHC1* | -2.5 | YLR298C U1 snRNP protein req. for pre-mRNA splicing |
| *CBP6* | -2.4 | YBR120C Translational activator of COB mRNA |
| *RRP8* | -2.4 | YDR083W similarity to hypothetical *S. pombe* protein |
| *RPC34* | -2.3 | YNR003C 34-kDa subunit of RNA polymerase III (C) |
| *RLP7* | -2.3 | YNL002C Significant sequence similarity to RPL7B |
| *RPA43* | -2.3 | YOR340C DNA-dependent RNA polymerase I subunit A43 |
| *RLP24* | -2.2 | YLR009W similarity to ribosomal protein L24.e.B |
| *RPB5* | -2.2 | YBR154C 25-kDa RNA polymerase subunit (common) |
| *RPB9* | -2 | YGL070C RNA polymerase II subunit |

**Table 3.8.** Genes involved in RNA processing or encoding subunits of RNA polymerases I, II, or III that are downregulated in *msi1Δ* cells

| Gene | Average Fold Δ | ORF and Description |
|---|---|---|
| *RPA34* | -5.1 | YJL148W RNA polymerase I subunit, not shared |
| *RNA15* | -3.1 | YGL044C cleavage and polyadenylation factor component |
| *RRP7* | -2.7 | YCL031C involved in rRNA processing, ribosome assembly |
| *ISY1* | -2.7 | YJR050W interacts with the spliceosome |
| *HCA4* | -2.5 | YJL033W putative RNA helicase |
| *BDF1* | -2.3 | YLR399C contains two bromodomains |
| *RPB4* | -2.3 | YJL140W fourth-largest subunit of RNA polymerase II |
| *RPC34* | -2.3 | YNR003C 34-kDa subunit of RNA polymerase III (C) |
| *YHC1* | -2.2 | YLR298C U1 snRNP protein required for mRNA splicing |
| *RRP3* | -2.2 | YHR065C encodes an RNA-dependent ATPase (DEAD box) |
| *RPA43* | -2.1 | YOR340C DNA-dependent RNA polymerase I subunit A43 |
| *MRT4* | -2.1 | YKL009W mRNA turnover 4 |
| *PRP11* | -2.1 | YDL043C snRNA-associated protein |
| *DRS1* | -2 | YLL008W putative ATP dependent RNA helicase |

**Table 3.9.** Misregulation of DNA damage response genes in CAF deletion mutants

| Gene | Average fold Δ for *asf1Δ* cells | Average fold Δ for *cac1Δ* cells | Average fold Δ for *msi1Δ* cells |
|---|---|---|---|
| *RNR3* | 6.5 | 3.2 | 1.9 |
| *RNR2* | 1.5 | 1.4 | 1.3 |
| *HUG1* | 9.5 | 7.5 | 2.1 |
| *UBI4* | 4.4 | 2.1 | 2.7 |

**Table 3.10.** Gene occupancy and transcriptional regulation by Asf1.

| Gene/ChIP primer set location[a] | Asf1 bound | Microarray fold change |
|---|---|---|
| *RNR2*/PR | + | 1.5 |
| *RNR3*/PR | + | 6.5 |
| *RNR4*/PR | + | NC |
| *RNR3*/DN | - | 6.5 |
| *CRT1*/PR | + | NC |
| *SAS10*/PR | + | -1.5 |
| *ACT1*/PR | + | NC |
| *CUP1*/PR | - | NC |
| *HHF2*/PR | + | -1.7 |
| *HTA1*/PR | + | NC |
| *HHT1*/PR | + | -1.7 |
| *ADH1*/PR | + | -2.4 |
| *SED1*/PR | + | NC |
| *PGK1*/PR | - | -2.3 |
| *PPH3*/PR | - | NC |
| *RPR1*/PR | + | 1.7 |
| *HSP30*/PR | - | NC |
| *DDR2*/PR | + | NC |
| MFα2/PR | - | 1.7 |
| *GPH1*/PR | + | 12.6 |

[a]PR, promoter; DN, downstream of coding region.

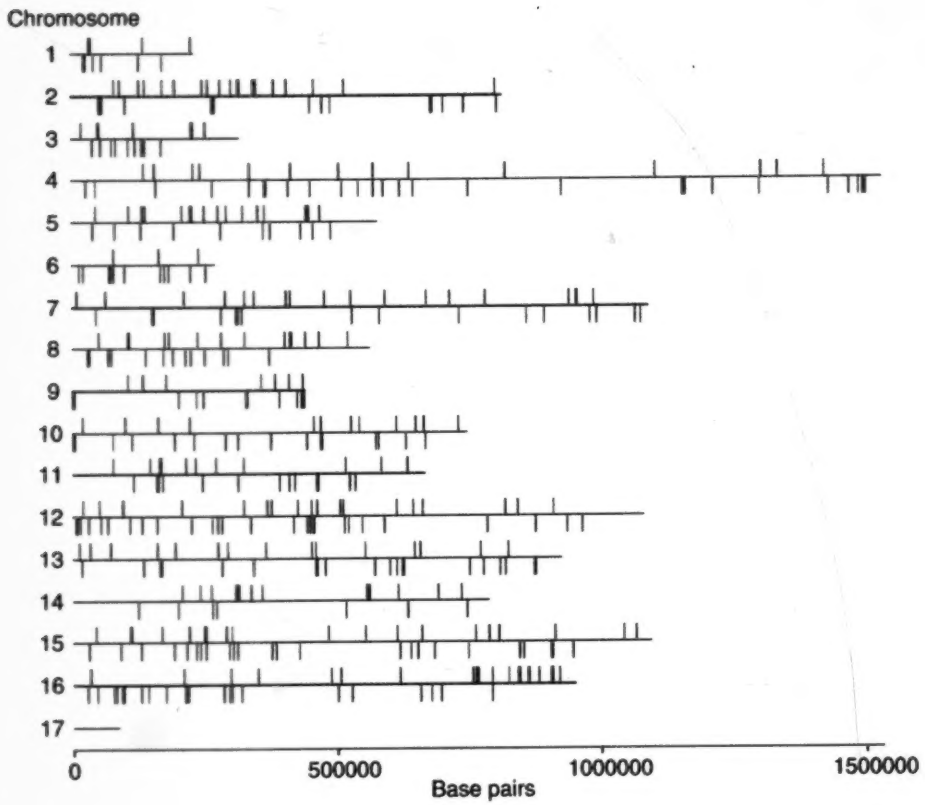


**Figure 3.1. Misregulated genes in *asf1Δ* cells are not clustered in specific chromosomal regions.** GeneSpring 5.1 map showing the physical location of genes which are induced (green) or repressed (red) by 2-fold or more in *asf1Δ* cells compared to wild type. In this representative experiment 536 genes differed by 2-fold or more in expression between the strains. The genes shown include a small number of tRNAs and Ty elements for which probes are present in Affymetrix S98 oligonucleotide arrays.

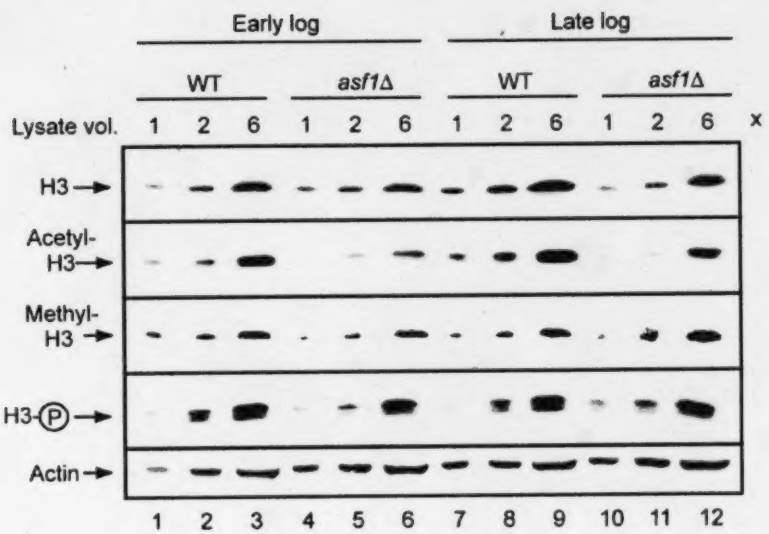


**Figure 3.2. Histone H3 metabolism is not significantly altered in *asf1Δ* cells.** Immunoblotting analysis of levels of bulk H3, K4/K9 di-acetylated H3, S10-phosphorylated H3, and K4-methylated H3 in wild type (WT) and *asf1Δ* cells grown to early or late log phase. The loading control is actin.

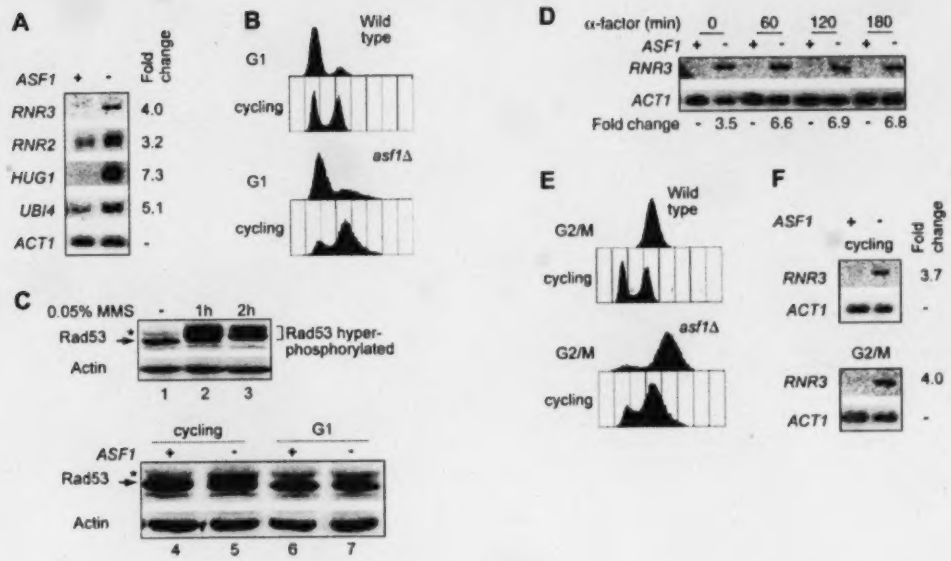


**Figure 3.3. Effect of *ASF1* deletion on expression of DNA damage inducible genes and modification state of Rad53. A.** Northern blotting analysis of selected genes in wild type and *asf1Δ* cells. Expression relative to wild type (Fold change) was normalized to the signal for *ACT1*. **B.** DNA content of cycling and $G_1$-arrested wild type and *asf1Δ* cells analyzed by flow cytometry. $G_1$ arrest was induced by culture in the presence of α-factor for 180 min. **C.** Rad53 modification state in wild type and *asf1Δ* cells. Rad53 was detected by immunoblotting in cycling wild type cells treated with MMS (lanes 1-3), and in proliferating (lanes 4, 5) and $G_1$-arrested (lanes 6, 7) wild type and *asf1Δ* cells. $G_1$ arrest was induced by culture in the presence of α-factor for 180 min. Equal protein loading of samples was confirmed by probing with a monoclonal antibody which recognizes actin. Arrow, hypophosphorylated Rad53; asterisk, minor band detected in all probings with the anti-Rad53 antibody used in this study. **D.** *RNR3* expression in cycling and α-factor-treated wild type and *asf1Δ* cells. The fold change in *RNR3* expression was calculated by normalization to *ACT1* recovery at the indicated times of culture in the presence of α-factor. **E.** DNA content of cycling and $G_2$/M-arrested wild type and *asf1Δ* cells analyzed by flow cytometry. **F.** *RNR3* expression in cycling and $G_2$/M-arrested cells. The fold change in *RNR3* expression was calculated by normalization to *ACT1* recovery.

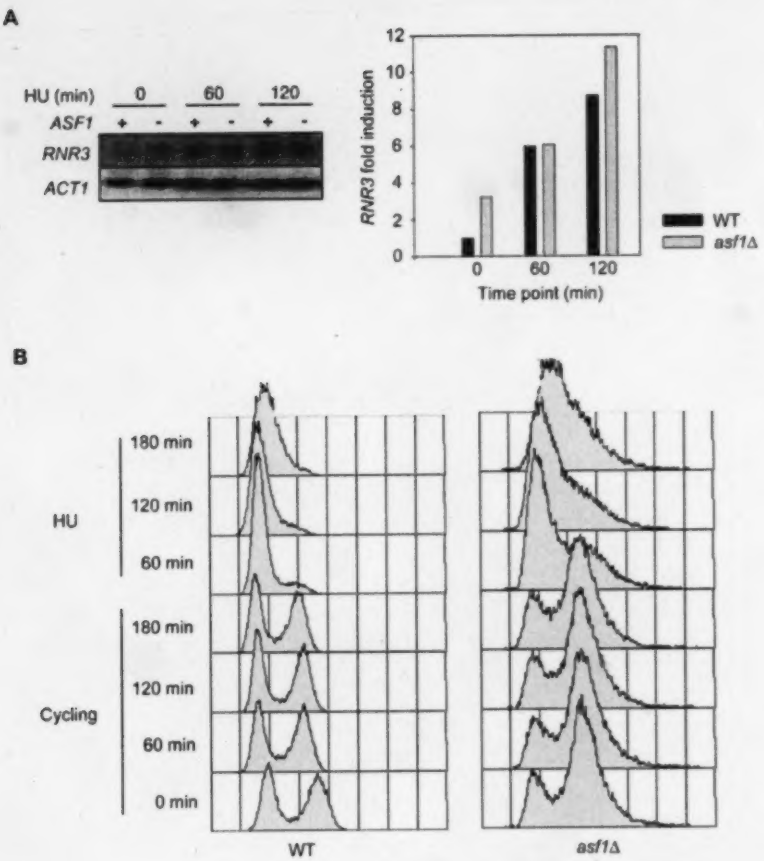


**Figure 3.4. Transcriptional and checkpoint responses to DNA damage are normal in *asf1Δ* cells. A.** Northern blotting analysis of *RNR3* expression in wild type and *asf1Δ* cells. Expression relative to wild type (Fold change) was normalized to the signal for *ACT1*. **B.** DNA content of cycling and S phase-arrested wild type and *asf1Δ* cells analyzed by flow cytometry. S phase arrest was induced by culture in the presence of hydroxyurea (HU) for the indicated time points. Florescence intensity on the X axis is plotted against cell number on the Y axis.

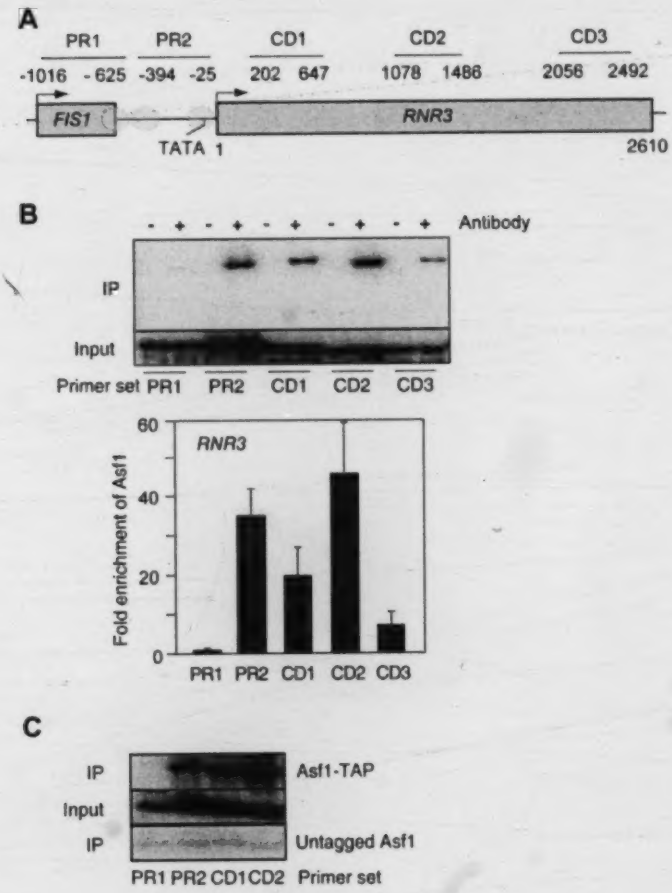


**Figure 3.5. *RNR3* occupancy by Asf1. A.** Location of primer sets used in ChIP experiments. The ovals represent nucleosomes which are present under non-inducing conditions and remodeled in response to genotoxic stress (Zhang and Reese, 2004a). **B.** Quantitative ChIP analysis of *RNR3* occupancy by Asf1-4HA. Following normalization using input values, fold enrichment of individual PCR products from the anti-HA immunoprecipitates was calculated relative to DNA recovery from mock immunoprecipitates (no antibody). The graph shows the mean of results from three independent experiments (+/- SD). **C.** ChIP analysis of *RNR3* occupancy by Asf1-TAP. Chromatin immunoprecipitates from tagged and untagged strains were analyzed using the indicated primer sets, and PCR products were detected by autoradiography.

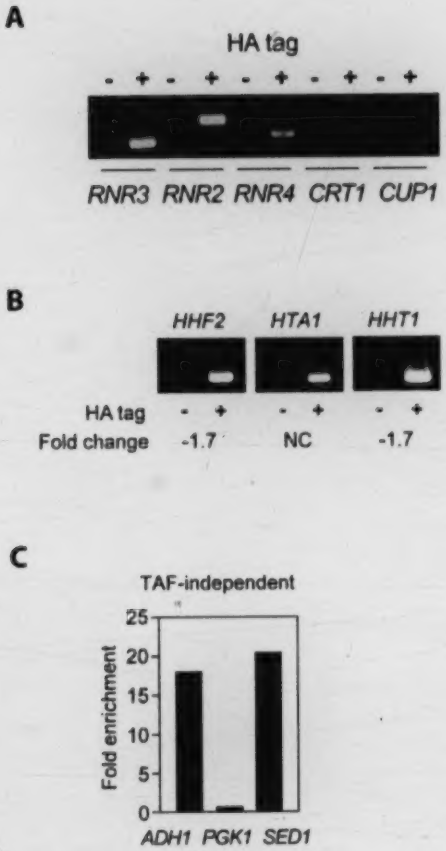


**Figure 3.6. Asf1 crosslinking to subsets of DNA damage response genes, histone genes, and $TAF_{II}$-independent genes. A.** ChIP analysis of Asf1-4HA at the promoters of the indicated DNA damage response genes in strains carrying untagged (-) or HA-tagged (+) Asf1. PCR products were detected by ethidium bromide staining. *CUP1* promoter; negative control. **B.** ChIP analysis of Asf1-4HA at the promoters of the indicated genes in strains carrying untagged (-) or HA-tagged (+) Asf1. PCR products were detected by ethidium bromide staining. The average fold change of expression of each gene (obtained by microarray analysis) for wild type versus *asf1Δ* cells is shown. **C.** Quantitative ChIP analysis of Asf1-4HA occupancy at three $TAF_{II}$-independent genes (Shen et al., 2003).

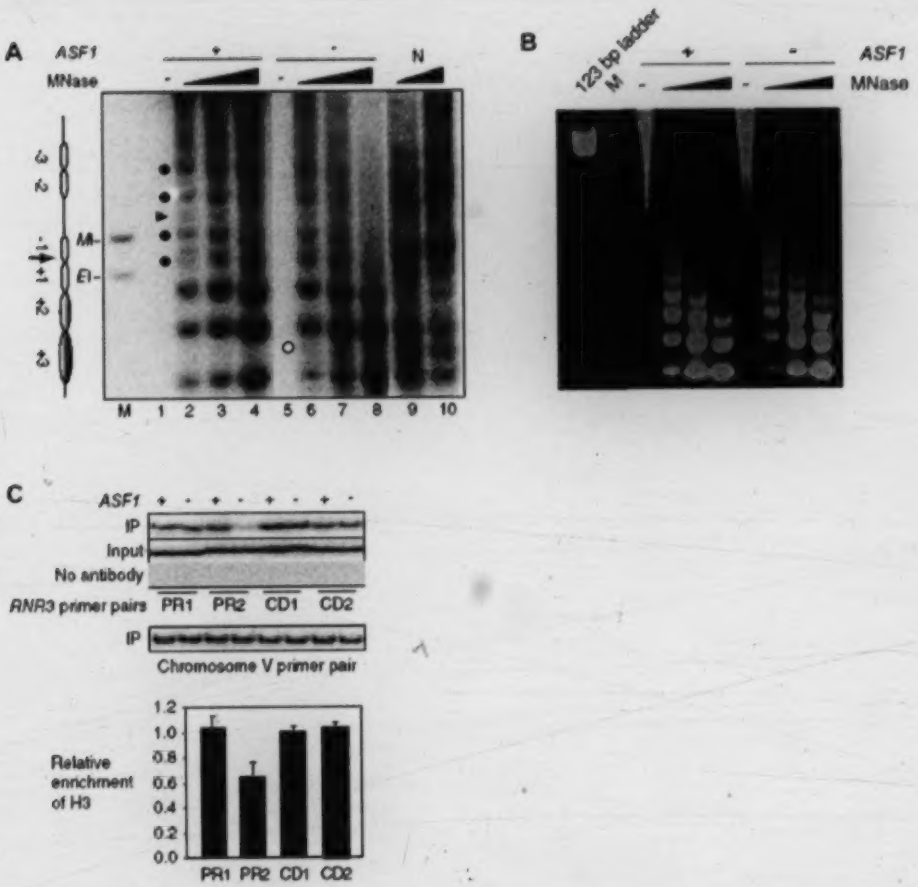


**Figure 3.7. Asf1-dependent chromatin remodeling at *RNR3*. A.** Chromatin structure of *RNR3*: response to *ASF1* deletion. Separate *Pst*I and *Mlu*I (*M*I), and *Pst*I and *Eag*I (*E*I) digests of genomic DNA were mixed and run in lane M. •, positions of altered sensitivity to digestion in *asf1Δ* cells; arrowhead, hypersensitive site not detected in digests of *asf1Δ* chromatin; ○, hypersensitive site not detected in digests of wild type chromatin; MNase, micrococcal nuclease; the arrow indicates the approximate start site of transcription. **B.** MNase digestion of bulk chromatin. Nuclei isolated from the indicated strains were digested with 0, 0.5, 1, and 2 U/ml MNase and the purified DNA products were resolved by 1.4% agarose-TAE gel electrophoresis. DNA staining is with ethidium bromide. Lane 1, 123 bp DNA ladder; Lane 2, lane M as in part A. **C.** Quantitative ChIP analysis of *RNR3* occupancy by histone H3. H3 occupancy in *asf1Δ* is expressed relative to recovery of H3-associated PCR products in the wild type strain (set to 1). The graph shows the mean of results from 4 independent experiments (+/- SD). *RNR3* DNA was not recovered in mock IPs ('No antibody') and H3 crosslinking to a chromosomal region which is devoid of genes was not affected by deletion of *ASF1* ('Chromosome V primer pair').

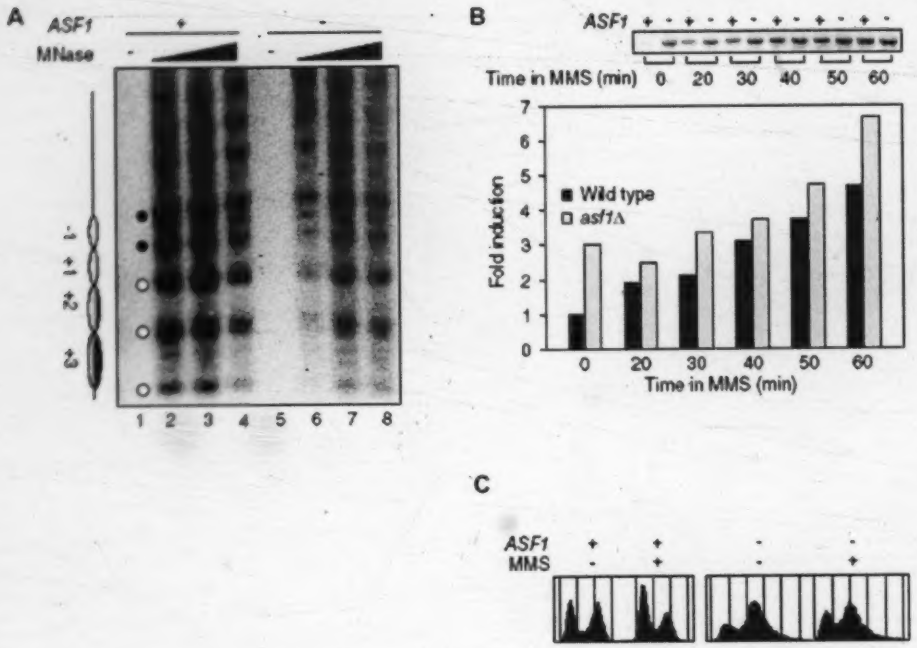


**Figure 3.8. Asf1-dependent chromatin remodeling and hyperinduction of *RNR3* in *asf1Δ* cells. A.** Chromatin structure of *RNR3*: response to genotoxic stress. Treatment with 0.05% MMS was for 2 h. Separate *Pst*I and *Mlu*I (*M*I), and *Pst*I and *Eag*I (*E*I) digests of genomic DNA were mixed and run in lane M. •, sites in the promoter which have increased sensitivity to digestion in *asf1Δ* cells; ○, hypersensitive sites flanking nucleosomes in the coding region; MNase, micrococcal nuclease. **B.** Northern blotting analysis of *RNR3* expression in wild type and *asf1Δ* cells treated with 0.1% MMS. *RNR3* signals shown in the insert were normalized to *ACT1* mRNA recovery (not shown) to generate the graph. **C.** Flow cytometry analysis of DNA content of the indicated strains before and after 1 h treatment with 0.1% MMS.

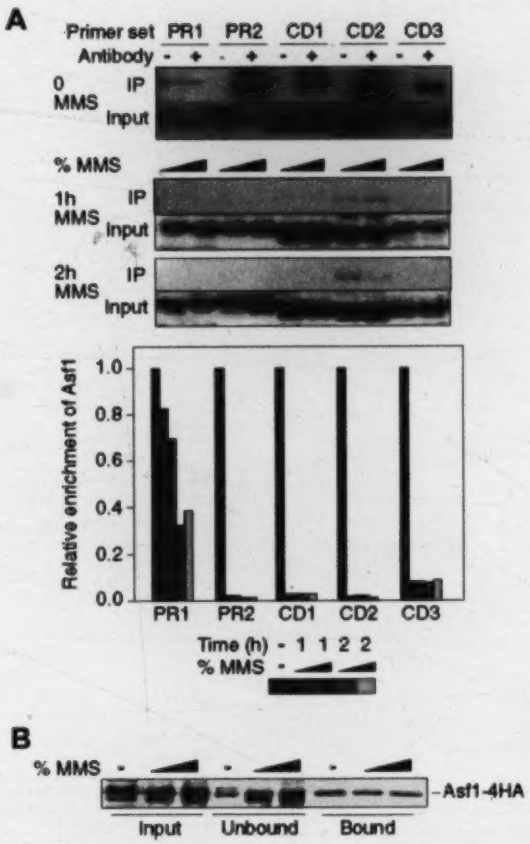


**Figure 3.9. Regulation of Asf1 occupancy at *RNR3*. A.** Quantitative ChIP analysis of Asf1-4HA crosslinking to *RNR3* before and after treatment with 0.05 or 0.1% MMS. Fold enrichment relative to recovery of individual PCR products from untreated cells (set to 1) was calculated. **B.** Anti-HA immunoblot of fractions from strains expressing HA-tagged Asf1 before (-) and after treatment with 0.05 or 0.1% MMS. The bound and unbound fractions from a ChIP whole cell extract were separated by immunoprecipitation using an anti-HA antibody.

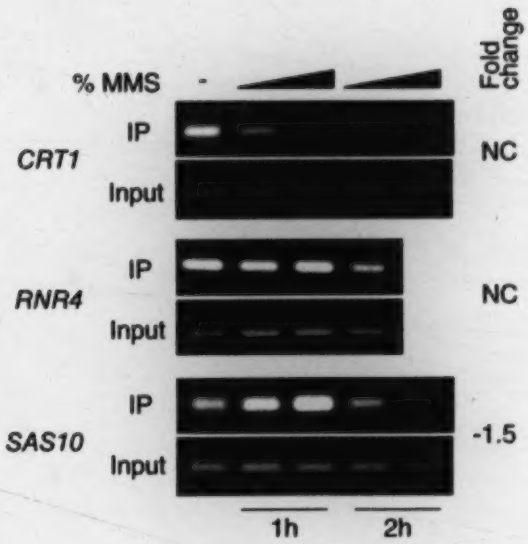


**Figure 3.10. Induction of DNA damage has relatively little effect on crosslinking of Asf1 to the promoters of *CRT1*, *RNR4*, and *SAS10*.** ChIP analysis of Asf1-4HA at the promoters of the indicated genes before and after treatment with 0.05 or 0.1% MMS. PCR products were detected by ethidium bromide staining. The average fold change of expression of each gene (obtained by microarray analysis) for wild type versus *asf1Δ* cells is shown.

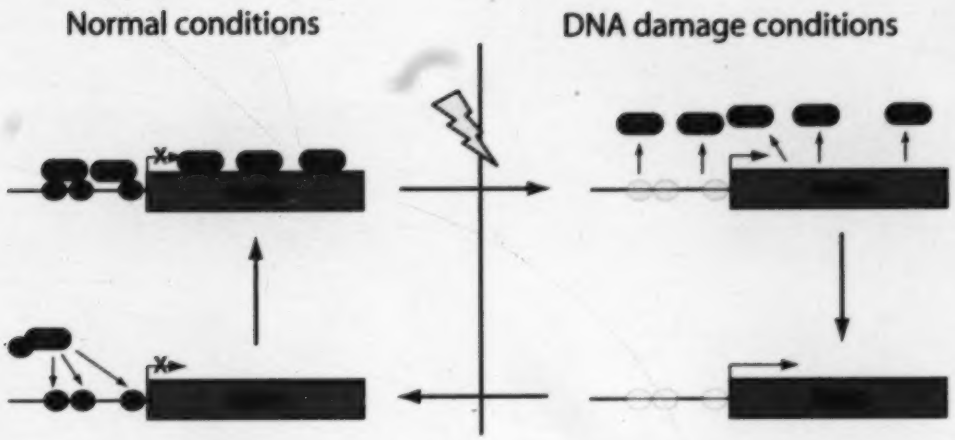


**Figure 3.11. Model for Asf1-dependent regulation of *RNR3* transcription and chromatin structure.** Under normal conditions Asf1 contributes to the assembly/stabilization of nucleosomes at the *RNR3* promoter, thus facilitating proper transcriptional repression of this gene. Asf1 also crosslinks to the *RNR3* promoter and coding region. When DNA damage occurs, Asf1 crosslinking to *RNR3* is significantly decreased. This inhibits H3/H4 deposition into nucleosomes at the *RNR3* promoter and contributes to derepression of transcription of this gene.

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# Chapter 4:

# Histone chaperone Asf1, histone variant Htz1, and chromatin remodeler Isw2 collaborate in regulation of the *RNR3* damage response gene

**Summary:**

The cellular response to DNA damage involves a highly regulated program of transcriptional reprogramming which includes derepression of *RNR3*. Proper chromatin structure and transcriptional regulation of this gene involves a myriad of proteins, including histone chaperone Asf1. Here we analyzed the role of various chromatin regulators in association of Asf1 with *RNR3*. We discovered that the function of Asf1 at *RNR3* is linked to metabolism of histone H2A variant Htz1, which also dissociates from *RNR3* and is required for normal *RNR3* induction in response to DNA damage. In addition, both the TFIID bromodomain-containing subunit, Bdf1, and chromatin remodeler, Isw2, contribute to regulation of Asf1 association with *RNR3*. In addition to its previously defined role in nucleosome positioning at *RNR3*, Isw2 is involved in regulation of the rate of transcriptional induction of this gene. Our identification of a genetic interaction between Asf1 and Isw2 during the response to DNA damage further implicates Isw2 in Asf1-dependent functions. Taken together, these results reveal that *RNR3* transcriptional regulation by Asf1 involves a histone variant, a subunit of the transcription machinery, and a chromatin remodeling enzyme.

**Introduction:**

Although the role of Asf1 in de novo nucleosome assembly has been well established, Asf1 may be used to modulate chromatin metabolism by contributing to events other than formation of new nucleosomes. We find that Asf1 functions in a targeted pathway of nucleosome assembly/stabilization which regulates transcription of the DNA damage inducible gene, *RNR3*. Direct regulation of transcription by Asf1 likely involves its interaction with other proteins. For example, human Asf1 physically interacts with the TAF1 ($TAF_{II}250$) subunit of transcription initiation factor TFIID, and in budding yeast Asf1 exists in a complex with TFIID and can directly bind to its bromodomain-containing Bdf1 and Bdf2 subunits (Chimura et al., 2002). *ASF1* and *BDF1* of yeast also interact genetically (Chimura et al., 2002).

Another gene with which *ASF1* interacts genetically is *HTZ1* (Krogan et al., 2003). It encodes the H2A.Z variant of histone H2A. Despite incorporation into only 5-10% of nucleosomes (Palmer et al., 1980; West and Bonner, 1980), H2A.Z plays a major role in transcriptional and cellular regulation (Ausio and Abbott, 2002). Htz1 preferentially associated with promoters under repressing conditions is also thought to poise promoter chromatin for activation (Larochelle and Gaudreau, 2003; Santisteban et al., 2000). Interestingly, both Htz1 and Bdf1 are components of the SWR1 chromatin remodeling complex required for the recruitment of Htz1 to specific chromosomal locations in vivo (Kobor et al., 2004; Krogan et al., 2003; Mizuguchi et al., 2004).

Following deposition of histones onto DNA by chaperones such as Asf1, ATPase-containing chromatin spacing complexes mobilize the nucleosomes to produce a regularly spaced nucleosomal array. Chromatin structure is dynamic, and remodeling factors play a specialized role in chromatin regulation through their ability to alter the positions of nucleosomes, thereby facilitating transcriptional repression or activation (Becker and Horz, 2002; Lusser and Kadonaga, 2003). *Drosophila* Asf1 has been detected in a complex which includes subunits of Brahma (Moshkin et al., 2002), an ATP-dependent chromatin remodeling machine involved in the activation and repression of transcription (Martens and Winston, 2003). Genetic interactions between fly *asf1* and the genes encoding several subunits of Brahma have also been documented (Moshkin et

al., 2002), suggesting the possibility that Asf1 may also interact with other chromatin remodeling enzymes.

Recent evidence from Zhang and Reese (2004a) has shown that the ISWI remodeling factor, Isw2, is involved in chromatin regulation at *RNR3*. The ISWI class of remodeling factors includes complexes that have been implicated in transcriptional regulation including both activation and repression, and also have been shown to play a role in chromatin assembly and maintenance of chromatin structure (Langst and Becker, 2001; Mellor and Morillon, 2004). There are two ISWI homologs in yeast, Isw1 and Isw2, both of which possess nucleosome-stimulated ATPase activity (Tsukiyama et al., 1999). The Isw2 complex is required for transcriptional repression at a number of loci, including those of early meiotic genes, where it functions in a pathway parallel to the Sin3–Rpd3 HDAC complex upon recruitment by Ume6 protein (Hughes et al., 2000; Goldmark et al., 2000; Fazzio et al., 2001). An Isw2-dependent mechanism regulates the access of transcriptional machinery to chromatin (Fazzio et al., 2001), and the chromatin structures of many genes are dependent on the Isw2 complex (Kent et al., 2001; Ruiz et al., 2003). Further evidence for a direct role in transcription comes from experiments showing that together with Isw1 and chromodomain protein Chd1, Isw2 is involved in transcriptional termination (Alen et al., 2002; Morillon et al., 2003). Because Isw2 is localized to *RNR3* under non-inducing conditions, we envisaged that Isw2 may function in a pathway of chromatin metabolism at *RNR3* which involves Isw2-dependent regulation of other *RNR3*-targeted factors.

Based on the observation that Asf1 directly participates in targeted chromatin dynamics to regulate transcription of the DNA damage-inducible *RNR3* gene, we attempted to identify proteins involved in targeting and regulation of Asf1 function at *RNR3*. Current knowledge of *RNR3* control suggests that numerous potential interacting partners may be involved, including transcriptional repressors, components of the transcriptional machinery, and chromatin remodeling complexes (Huang et al., 1998; Li and Reese, 2000; Li and Reese, 2001; Sharma et al., 2003; Zhang and Reese, 2004a,b). In this chapter, we present evidence in favor of roles for Htz1, Bdf1, and Isw2 in Asf1-dependent steps of chromatin metabolism at *RNR3*.

**Results:**

**Asf1 associates with *RNR3* independently of the Crt1 repressor**

Crt1 plays a central role in the regulation of *RNR3* by virtue of its ability to recruit a corepressor complex, comprised of Tup1 and Ssn6, to the *RNR3* promoter (Huang et al., 1998; Li and Reese, 2001; Sharma et al., 2003). Tup1 is critically important for repression, partly because its interaction with histones H3/H4 controls the chromatin structure of *RNR3*. Because Asf1 does not bind to DNA and Crt1 is already known to recruit a histone-binding factor (Tup1) involved in chromatin metabolism at *RNR3*, we tested if, under normal conditions, Crt1 is also necessary and sufficient for association of Asf1 with *RNR3*. Asf1 occupancy of *RNR3* and adjacent DNA was probed in wild type and *crt1*Δ cells by ChIP. The location of PCR primer sets spanning the promoter and coding region of *RNR3* are indicated in Figure 4.1, A. Recovery of *RNR3* DNA was essentially identical in these strains (Fig. 4.1, B). Therefore Crt1 is not uniquely required for specific association of Asf1 with *RNR3*. This idea is supported by our observation that soluble GST-tagged Asf1 expressed from a high-copy plasmid vector under control of the *CUP1* promoter does not coimmunoprecipitate with Ssn6 or Tup1 (Fig. 4.1, C). Furthermore, Asf1-4HA does not coimmunoprecipitate with Tup1 (in this case both proteins are expressed at endogenous levels; Fig. 4.1, C). Most likely interaction of Asf1 with other factor/s bound to *RNR3* is important for specific localization of Asf1 to *RNR3*. As a potential Asf1-localizing factor, the homeodomain protein Yox1 is in the same functional category as Crt1; it is found at *RNR3* under non-inducing conditions (Horak et al., 2002) and represses transcription at genes where its role has been specifically analyzed (Pramila et al., 2002). Asf1 association with *RNR3*, however, was not sensitive to *YOX1* deletion (Fig. 4.1, D).

Deletion of *CRT1* is associated with substantially greater derepression of *RNR3* under normal conditions (greater than 27-fold; Huang et al., 1998; J. Williams and M. Schultz, unpublished data) than is deletion of *ASF1* (3.5-6.3 fold; Chapter 3). This data suggests either that Asf1 on its own makes a relatively minor contribution to repression of *RNR3*, or that another histone chaperone can partially substitute for Asf1 in the *asf1*Δ mutant. The latter argument has been used repeatedly to explain why deletion of an individual histone chaperone such as CAF-1 does not have a dramatic effect on

chromatin metabolism in vivo (reviewed in Ridgway and Almouzni, 2000). Regardless of the relative contribution of Crt1 and Asf1 to regulation of *RNR3*, the result of the experiment in Figure 4.1, A and our confirmation that *RNR3* transcription is strongly induced in *crt1Δ* cells (J. Williams and M. Schultz, unpublished data) clearly indicates that the process of transcription is compatible with Asf1 occupancy of *RNR3*. Therefore it is highly unlikely that transcription per se is responsible for dissociation of Asf1 when cells experience genotoxic stress.

**Histone H2A variant Htz1 is present at *RNR3* and crosslinking of Asf1 to *RNR3* is perturbed in *htz1*Δ and *bdf1*Δ cells**

We have begun to examine how the association of Asf1 with *RNR3* might involve other chromatin proteins. Three observations encouraged our present focus on histone H2A variant Htz1. First, although it does not bind to specific DNA sequences, Htz1 has been localized to specific genes (Kobor et al., 2004; Krogan et al., 2003; Larochelle and Gaudreau, 2003; Meneghini et al., 2003; Mizuguchi et al., 2004; Santisteban et al., 2000). Second, *ASF1* interacts genetically with *HTZ1* (Krogan et al., 2003). Finally, both *asf1*Δ and *htz1*Δ mutants are sensitive to MMS (Krogan et al., 2003; see Fig. 4.7).

Initially we used ChIP to explore the possibility that Htz1 is associated with the *RNR3* locus. For this experiment we used a published strain that expresses TAP-tagged Htz1 in place of the wild type gene (Ghaemmaghami et al., 2003) and immunoprecipitation was performed with rabbit IgG. To test if TAP-tagging interferes with Htz1 function, wild type, *htz1Δ* and HTZ1-TAP strains were plated on a standard synthetic growth medium with no additive, with 20 mM caffeine, a compound that has pleiotropic effects in the cell, or with 150 mM hydroxyurea (Fig. 4.2, A). *htz1Δ* cells have a growth defect on synthetic medium which is exacerbated in the presence of caffeine and hydroxyurea, as expected (Mizuguchi et al., 2004). None of these growth defects was observed in cells expressing Htz1-TAP. Therefore, although the TAP tag is larger than Htz1 itself, Htz1-TAP appears to be fully functional in vivo. A similar conclusion has been reached by others (unpublished data reported in Krogan et al., 2003). Crosslinking to *RNR3* was not observed in control ChIP experiments using a strain expressing untagged Htz1 (J. Williams and M. Schultz, unpublished data). In experiments using the TAP-tagged Htz1 strain, on the other hand, strong crosslinking was

obtained over the coding region of *RNR3* (Fig. 4.2, B). Crosslinking to the promoter was detectable, but approached background levels. We conclude that Htz1 is associated with *RNR3* under normal repressive conditions and that it is enriched in the coding region. A similar distribution of Htz1 has been reported for other genes. For example, Htz1 is enriched in the coding region of the *GAL1* gene under conditions that repress its transcription (Krogan et al., 2003).

Because Htz1 normally associates with the coding region of *RNR3*, where Asf1 is also found, we tested if crosslinking of Asf1 to *RNR3* requires Htz1. For this experiment *HTZ1* was deleted in the strain which expresses Asf1-4HA. In the absence of Htz1, crosslinking of Asf1 to the coding region of *RNR3* declines (Fig. 4.2, C). The effect is restricted to the 5' end of the ORF, and is modest. Unexpectedly, at the promoter where Htz1 is virtually undetectable, Asf1 crosslinking is strongly inhibited in *htz1*Δ cells. Therefore co-localization of Asf1 and Htz1 to the promoter is not required for *HTZ1* deletion to have an effect on association of Asf1 with *RNR3* at this location. Our results are compatible with a model in which an Htz1-dependent configuration of chromatin at the 5' end of the coding region is important for promoter crosslinking of Asf1. In other words, the disposition of Asf1 in the promoter may be dictated in part by the molecular composition of chromatin at its downstream boundary. Alternatively, deletion of *HTZ1* could have indirect effects which impinge on Asf1 located in the promoter but not the coding region of *RNR3*. We do not favor this idea because under normal conditions deletion of *HTZ1* does not affect bulk expression of Asf1 (Fig. 4.2, D) or *RNR3* transcription (Fig. 4.4, A).

We also tested whether another SWR1 complex component, bromodomain factor Bdf1, is required for association of Asf1 with *RNR3*. Like Htz1, Bdf1 has been localized to specific genes by ChIP (Matangkasombut and Buratowski, 2003; Ladurner et al., 2003). *ASF1* interacts genetically with *BDF1*, and Bdf1 directly binds to Asf1 in vivo (Chimura et al., 2002). Both being subunits of the SWR1 chromatin remodeling complex, Bdf1 and Htz1 are likely to have some shared functions in chromatin metabolism (Krogan et al., 2003; Mizuguchi et al., 2004; Kobor et al., 2004). Comparison of Asf1-4HA occupancy across *RNR3* between wild type and *bdf1*Δ strains by quantitative ChIP revealed a reduction in association of Asf1 with *RNR3* in *bdf1*Δ

cells, but to a lesser extent overall than in *htz1*Δ cells. *BDF1* deletion mainly affected Asf1 association with the 5' coding region of *RNR3* (Fig. 4.3, A), to about the same extent as *HTZ1* deletion affects association with the 5' coding region (Fig. 4.2, C). As for *HTZ1*, deletion of *BDF1* does not affect bulk expression of Asf1 (Fig. 4.3, B). We conclude that promoter association of Asf1 with *RNR3* is strongly dependent on Htz1, and that Htz1 and Bdf1 make a similar less pronounced contribution to Asf1 association with the 5' region.

**A role for Htz1 in DNA damage regulation of *RNR3* transcription**

We next determined whether like Asf1, Htz1 is also required for transcriptional regulation of *RNR3*. In published microarray studies, *RNR3* is not misexpressed in cycling *htz1*Δ cells (Meneghini et al., 2003; Mizuguchi et al., 2004). This finding was confirmed in a direct comparison of *RNR3* expression in wild type, *asf1*Δ, *htz1*Δ and *asf1*Δ *htz1*Δ cells by Northern blotting. A representative experiment is shown in Figure 4.4, A, where *RNR3* expression was 6.9-fold induced in *asf1*Δ cells but essentially unaffected in *htz1*Δ cells, and derepression of *RNR3* was similar in *asf1*Δ and *asf1*Δ *htz1*Δ cells. This result seems to rule out a substantial role for Htz1 in Asf1-dependent repression of *RNR3*. Our findings do not, however, exclude the possibility that Asf1 and Htz1 contribute to the specific steps in gene regulation in other contexts. Indeed, there is remarkable overlap between the gene expression signatures of *asf1*Δ and *htz1*Δ mutants (see Table 4.2). Of 105 genes that are induced in *htz1*Δ cells, 70% were also induced in the *asf1*Δ mutant (Fig. 4.4, B; $\chi^2$ test of independence, $P = 4.3 \times 10^{-8}$). This overlap suggests that under normal conditions repression of some genes involves both Asf1 and Htz1. Furthermore, it is possible that the contribution of Htz1 to the regulation of some Asf1-dependent genes is not revealed in this comparison because at some loci Htz1 functions only in the context of physiological induction. This might account for the failure to detect an effect of *HTZ1* deletion on the basal (un-induced) expression of several genes which are occupied by Htz1 in vivo (Krogan et al., 2003). In light of these considerations, we explored the possibility that Htz1 is involved in transcriptional induction of *RNR3* in response to DNA damage. Expression of *RNR3* was examined in wild type, *asf1*Δ, *htz1*Δ and *asf1*Δ *htz1*Δcells exposed to 0.05% MMS for 1 or 2 hours. A

representative experiment is shown in Figure 4.4, C. Quantitative analysis of the data reveals that *RNR3* is modestly hyperinduced in *htz1*Δ cells at 1 h following addition of MMS (47% more than in wild type). The level of *RNR3* expression in *htz1*Δ cells treated for 1 h is only attained in wild type cells after 2 h of exposure to MMS. Considering that *RNR3* is repressed under normal conditions in *htz1*Δ cells, these results suggest that an Htz1-dependent mechanism limits the rate of *RNR3* induction. We propose that Htz1 functions in a pathway that regulates total transcriptional output of *RNR3* mRNA by modulating the rate of transcription during the early phase of the DNA damage response.

Consistent with this proposal, crosslinking of Htz1-TAP to *RNR3* is also responsive to genotoxic stress. Specifically, association of Htz1 with *RNR3* decreases in a dose-dependent fashion when cells are exposed to MMS (Fig. 4.5, A), declining in the coding region to approximately 15% of the level observed in untreated cells. Because bulk expression of Htz1 does not change in cells exposed to MMS (Fig. 4.5, B), we propose that reduced crosslinking of Htz1 reflects a change in chromatin organization which impinges on Htz1 molecules present at *RNR3*.

The results outlined above support the hypothesis that association of both Asf1 and Htz1 with *RNR3* is regulated by genotoxic stress signals. Independent evidence for DNA damage regulation of Htz1 was obtained in experiments which monitored coimmunoprecipitation of Htz1 with Asf1 over-produced in untreated and MMS-treated yeast cells. In this situation coimmunoprecipitation of Asf1 with Htz1 could be due to their direct interaction or their indirect association as components of a complex with other subunits. Regarding the latter scenario, we reasoned that indirect association could occur in the context of the SWR1 chromatin remodeling complex which contains Htz1 and Bdf1 (Kobor et al., 2004; Krogan et al., 2003; Mizuguchi et al., 2004). This complex functions in vivo to exchange nucleosomal H2A for Htz1, by a mechanism involving its Swr1 catalytic subunit. For this experiment a high copy plasmid bearing copper-inducible GST-Asf1, or GST alone, was transformed into *asf1*Δ strains in which the endogenous *HTZ1* or *SWR1* gene had been TAP-tagged at its C-terminus. Expression of GST-Asf1 or GST was induced by growing the cells in 0.6 mM $CuSO_4$ for 40 min. TAP pull-downs from whole cell extracts were probed by immunoblotting using an anti-GST antibody. Two negative controls validated the specificity of this assay. First, GST-Asf1

could not be detected in anti-TAP pull-downs from strains which expressed GST-Asf1 but no TAP-tagged protein (Fig. 4.6, A). Second, no species which co-migrates with GST-Asf1 is detected in TAP pull-downs from strains expressing GST on its own (Fig. 4.6, B, odd-numbered lanes). As a positive control we tested for the previously demonstrated physical interaction between Asf1 and Bdf1 (Chimura et al., 2002). Under the conditions that physical association between GST-Asf1 and Bdf1-TAP was detected, we were also able to copurify GST-Asf1 with Htz1-TAP or Swr1-TAP (Fig. 4.6, B). Despite the fact that Bdf1 is 2.9-fold more abundant than Htz1 (Ghaemmaghami et al., 2003), roughly the same amount of GST-Asf1 was recovered in Htz1-TAP and Bdf1-TAP immune complexes. Somewhat less Swr1-TAP than either Bdf1-TAP or Htz1-TAP is recovered in GST-Asf1 immune complexes (Swr1 is about 12-fold less abundant than Bdf1 and 4-fold less abundant than Htz1). These interactions were not due to nucleic acid bridging, as the protein extracts were treated with RNase and DNase prior to immunoprecipitation. Neither were they due to interaction of the TAP tag itself with GST, as neither GST nor GST-Asf1 were detected in the bound fraction following immunoprecipitation of the histone H2A-TAP tagged protein (Fig. 4.6, C). We conclude that Asf1 is capable of stable association with Htz1 and Swr1 in vivo. More importantly, the interaction of overexpressed GST-Asf1 with Htz1-TAP is modulated by DNA damage signals. Specifically, the recovery of soluble Asf1-Htz1 immune complexes increases substantially when cells are treated with MMS (Fig. 4.6, B, compare lanes 6 and 8). This result confirms that Htz1 interacts with Asf1 in a biochemical pathway which is responsive to DNA damage signals, and the increased recovery of soluble complexes containing both Asf1 and Htz1 is consistent with our evidence in favor of the idea that a subpopulation of Htz1 molecules is mobilized from the chromatin fraction to the soluble fraction upon DNA damage (Fig. 4.5, A).

We sought genetic evidence in favor of the idea that Asf1 and Htz1 may interact during the response to DNA damage by performing genetic epistasis analysis. Separate studies have reported that *asf1Δ* and *htz1Δ* mutants are sensitive to thermal stress and to MMS, but the phenotypes of these strains have not been directly compared (Le et al., 1997; Singer et al., 1998; Tyler et al., 1999; Mizuguchi et al., 2004). We performed this comparison by plating serial dilutions of *asf1Δ*, *htz1Δ* and *asf1Δ htz1Δ* cells on rich

medium at 30°C and 37°C in the presence or absence of MMS. Plates were photographed after 2 d growth. This experiment revealed that *asf1Δ* cells are more sensitive than *htz1Δ* cells to thermal stress and to MMS (Fig. 4.7). Genetic interaction of *ASF1* and *HTZ1*, as previously reported (Krogan et al., 2003), was also evident; both single mutants grow faster than the *asf1Δ htz1Δ* double mutant on rich medium. This growth defect of *asf1Δ htz1Δ* cells, however, is not exacerbated when MMS is included in rich plating medium up to a concentration of 0.02% (Fig. 4.7). This is in agreement with Asf1 and Htz1 having an epistatic relationship in terms of the response to DNA damage. For example, Asf1 and Htz1 may participate in the same pathway of induction of genes whose products are important for survival of DNA damage. In summary, *asf1Δ* and *htz1Δ* cells have similar but not identical phenotypes and their combined deletion no more seriously affects growth rate in the presence of MMS than it affects growth in general.

**Association of Asf1 with the *RNR3* promoter involves Isw2**

We now extended our analysis of how the association of Asf1 with *RNR3* might involve other chromatin proteins by focusing on the chromatin remodeling factor, Isw2. Many aspects of Asf1-dependent *RNR3* regulation resemble the recently reported effects of regulation of this gene by Isw2 (Zhang and Reese, 2004a, b). First, deletion of either Asf1 or Isw2 causes modest derepression of *RNR3*. Second, both Asf1 and Isw2 can be crosslinked to the *RNR3* promoter and coding region. Third, both proteins are required for the establishment of repressive chromatin organization at this gene, and this appears to involve promoter histone dynamics (Zhang and Reese, 2004a; Chapter 3). The overall similarity of *RNR3* regulation by Asf1 and Isw2 prompted an investigation of the possibility of a relationship between these proteins during *RNR3* regulation by performing ChIP analysis using wild type and *isw2Δ* strains expressing HA-tagged Asf1. The location of primer sets spanning the *RNR3* promoter and coding region are indicated in Figure 4.8, A.

Asf1 was readily detected across *RNR3* in *isw2Δ* cells (Fig. 4.8, B). Surprisingly, the level of Asf1 crosslinking at the *RNR3* promoter was reproducibly increased by approximately 2-fold in the absence of *ISW2* compared to wild type. This effect is highly specific for the promoter; at three positions in the coding region of *RNR3* deletion of

*ISW2* has essentially no effect on Asf1 cross-linking (primer sets CD1, CD2 and CD3 in Fig. 4.8, B). Global misregulation of Asf1 protein expression did not account for this effect since bulk expression of Asf1 was not altered in the *ISW2* null strain when compared to the wild type strain (Fig. 4.8, C). Control reactions using immunoprecipitated template DNA isolated from untagged strains also revealed that the ChIP signal was dependent on specific immunoprecipitation of Asf1-4HA (Fig. 4.8, B, 'No tag' panel).

The possibility of a reciprocal relationship in which Isw2 crosslinking to *RNR3* is dependent on Asf1 was determined in order to further dissect the relationship between Asf1 and Isw2 at this gene. For example, it may be that Asf1-dependent alterations in chromatin structure at this gene affect Isw2 occupancy. This may result in either an increase or decrease in Isw2 association. However, it may be the case that Isw2 occupancy at *RNR3* is independent of Asf1 function. In order to discriminate between these possibilities, we compared the occupancy of *RNR3* by TAP-tagged Isw2 in wild type and *asf1Δ* cells. The results presented in Figure 4.9, A, indicate that *ASF1* deletion did not affect the level of Isw2 crosslinking to *RNR3*, and *ASF1* deletion had no effect on bulk expression of Isw2 (Fig. 4.9, B). Therefore, Asf1 is not uniquely required for association of Isw2 with *RNR3*. This idea is consistent with our observation that HA-tagged Asf1 did not coimmunoprecipitate with Isw2-TAP (Fig. 4.9, C). These results also demonstrate that Isw2 remains present at *RNR3* during derepression conditions associated with *ASF1* deletion. On a similar note, Zhang and Reese (2004a) reported that Isw2 crosslinking is not affected upon deletion of the *SSN6* or *TUP1* repressors. Taken together, these ChIP results reveal that although Isw2 crosslinking to *RNR3* appears to be independent of Asf1, crosslinking of Asf1 to the *RNR3* promoter is regulated in an Isw2-dependent manner.

**Isw2 is required for proper regulation of *RNR3* transcriptional induction during the response to DNA damage**

We used Northern blot analysis in an attempt to gain further insight into the contributions made by Asf1 and Isw2 during transcriptional regulation of *RNR3*. Expression levels of *RNR3* were quantitated in wild type, *asf1Δ*, *isw2Δ*, and *asf1Δisw2Δ* cells before and after treatment with 0.05% MMS for 1 or 2 hours. A representative

experiment summarizing the results from four independent experiments is shown in Figure 4.10, A (results between replicates varied by less than 10%). Under normal conditions, *RNR3* was induced by approximately 2-fold in the *isw2Δ* strain, and the level of *RNR3* hyperinduction observed in the *asf1Δ* single mutant was similar to the level in the *asf1Δisw2Δ* double mutant (about 4-fold). Flow cytometry analysis indicated that the previously documented S phase and $G_2$/M defects in cell cycle progression observed in an *asf1Δ* mutant were not affected by loss of *ISW2* (Fig. 4.10, B). MMS treatment resulted in *RNR3* induction in all strains tested (Fig. 4.10, A). Despite only a modest level of *RNR3* induction in the *ISW2* null cells under benign conditions, we found that the transcriptional response of *RNR3* was perturbed in the absence of Isw2 during the response to DNA damage. As in MMS-treated *asf1Δ* cells, *RNR3* was reproducibly hyperinduced in the absence of Isw2 during the response to DNA damage (Fig. 4.10, A). Similar perturbation of *RNR3* expression in *isw2Δ* cells was reported by Zhang and Reese (2004b). Quantitation revealed that *RNR3* was hyperinduced in the *isw2Δ* strain when compared to wild type, *asf1Δ*, or *asf1Δisw2Δ* double mutant at both timepoints of MMS treatment, (Fig. 4.10, A). A second effect of *ISW2* deletion on DNA damage regulation of *RNR3* was to reduce its fold stimulation in the presence of MMS. Quantitation of this and previous experiments (Fig. 3.8, B; Fig. 4.4, C; Fig. 4.10, A) has shown that loss of Asf1 causes on overall reduction in the fold stimulation of *RNR3* expression in the presence of MMS (3.5-fold lower in *asf1Δ* than wild type cells in Fig. 4.10, A). Similarly, in Figure 4.10, A, the level of *RNR3* induction due to MMS treatment is lower in *isw2Δ* than wild type cells (approximately 11-fold). This value falls in between the fold of induction observed in wild type cells (17-fold) and the fold of induction in *asf1Δ* cells (5-fold in this experiment). We did not observe a synergistic effect on *RNR3* expression in the *asf1Δisw2Δ* double mutant (Fig. 4.10, A). The level of *RNR3* derepression in the double mutant strain was similar to level observed in the *isw2Δ* mutant at the 1 h timepoint of MMS treatment, and by the later 2 h timepoint of DNA damage, the level of *RNR3* derepression in the *asf1Δ isw2Δ* double mutant was very similar to the level in wild type cells. Collectively, our results suggest that Isw2 is required for normal transcriptional induction of *RNR3* during the DNA damage response.

These results are consistent with a model in which both Asf1 and Isw2 are involved in *RNR3* transcriptional regulation. Under normal conditions, both proteins contribute to establishment of a repressive state which involves alterations in chromatin structure at *RNR3* (Fig. 3.7, A; Zhang and Reese, 2004a). In response to DNA damage, both Asf1 and Isw2 are involved in *RNR3* transcriptional induction.

***ASF1* and *ISW2* show a genetic interaction under DNA damage conditions**

The requirement for both Asf1 and Isw2 in regulation of *RNR3* suggested the possibility of a more global interaction between these proteins during the response to genotoxic stress (Zhang and Reese, 2004a; Chapter 3). We therefore sought genetic evidence in favor of the idea that Asf1 and Isw2 may interact during the response to DNA damage. Genetic epistasis analysis of mutant strains was performed by plating on rich medium in the presence of varying concentrations of the MMS. We tested growth at both 30°C and 37°C, as combination of *ISW2* deletion with *ISW1* and *CHD1* deletions has been shown to cause sensitivity to temperature stress (Tsukiyama et al., 1999). Deletion of *ASF1* was combined with deletion of *ISW2* and the sensitivity of two independent isolates of each mutant strain to elevated temperature and DNA damage was directly compared. It was expected that a synergistic genetic interaction would be observed if Asf1 and Isw2 were components of parallel functional pathways. On the other hand, if these proteins function in the same genetic pathway we expected that the *asf1Δ isw2Δ* double mutant would show no additional sensitivity to MMS when compared to the *asf1Δ* single mutant. Growth of *asf1Δ* cells, *isw2Δ* cells, or *asf1Δ isw2Δ* double mutant cells was not significantly perturbed on rich medium in the absence of drug when compared to the wild type strain (Fig. 4.11, A). As previously observed, *asf1Δ* cells were sensitive to damage induced by MMS treatment (Le et al., 1997; Singer et al., 1998; Tyler et al., 1999; Fig. 4.7). Loss of Isw2 function did not render cells hypersensitive to MMS or cause the *asf1Δ* strain to become sensitive to temperature stress (Fig. 4.11, A). To our surprise, *ISW2* deletion partially ameliorated the MMS-sensitivity of *asf1Δ* cells (Fig. 4.11, A; compare growth between *asf1Δ* and *asf1Δ isw2Δ* in the presence of 0.0075% or 0.15% MMS, especially at 30°C). This result suggests that an Isw2-dependent function has negative consequences in the absence of Asf1 under conditions of genotoxic stress. Elevated temperature did not have a significant impact on the growth rate differences

observed between *asf1Δ* cells and *asf1Δ isw2Δ* cells during DNA damage (Fig. 4.11, A). We extended this experiment to include a streaking assay on rich medium supplemented with intermediate MMS concentrations. Figure 4.11, B clearly shows that the survival of *asf1Δ isw2Δ* cells during MMS treatment was enhanced when compared to the *asf1Δ* single mutant. These results suggest that loss of *ISW2* stimulates the survival of *asf1Δ* cells during genotoxic stress.

Based on the partial suppression of *asf1Δ* sensitivity to MMS by deletion of *ISW2*, it was expected that overexpression of Isw2 would increase the DNA damage-sensitivity of an *asf1Δ* strain. To address this possibility, high copy number vectors without an insert or expressing Isw2p tagged at the N-terminus with GST were used to transform wild type and *asf1Δ* cells. Cell growth was monitored by plating serial dilutions of two independent isolates of each genotype on minimal medium in the presence or absence of varying MMS concentrations. Plating results presented in Figure 4.12, A, show that overexpression of GST-Isw2 caused a synthetic sick phenotype in *asf1Δ* cells but not in the wild type cells during MMS treatment, or in either cell type transformed with vector alone. This difference in growth rate was not due to differential expression of GST-Isw2, as copper-induced protein expression levels were similar in the wild type and *asf1Δ* cells (Fig. 4.12, B). At an MMS concentration of 0.01%, GST-Isw2 overexpression in *asf1Δ* reproducibly caused impaired growth when compared to growth of *asf1Δ* cells transformed with vector alone. The streaking comparisons on 0.01% and 0.015% MMS presented in Figure 4.12, C, confirm and strengthen this result. Therefore, combination of *ASF1* deletion with overexpression of Isw2 results in a synergistic hypersensitivity to MMS. It appears that Isw2 plays a negative role in survival of *asf1Δ* cells during MMS treatment. Taken together, these data suggest a functional interaction between Asf1 and Isw2 is involved in survival of DNA damage, with these proteins having opposing positions during this response. Therefore, in addition to the roles played by Asf1 and Isw2 in chromatin structure and transcriptional regulation of the *RNR3* DNA damage response gene, they also functionally interact under physiological conditions that regulate expression of this gene.

**Discussion:**

**What molecules function in collaboration with Asf1 at *RNR3*?**

Regulation of *RNR3* by Asf1 is likely dependent on its interaction with histones, components of the transcriptional apparatus, and components of the DNA damage signaling machinery. We initially focused our attempt to identify proteins that function in Asf1-dependent regulation of *RNR3* on components of the transcriptional machinery. Because *RNR3*-associated Asf1 normally represses transcription, we tested if one or more of the repressors already known (or likely) to function at *RNR3* is necessary and sufficient for Asf1 localization. Association of Asf1 with *RNR3*, however, did not exclusively require either the Crt1-Tup1/Ssn6 complex or the Yox1 repressor (Fig. 4.1). This outcome raises the possibility that Yox1 and Crt1 function redundantly in the recruitment and/or retention of Asf1 at *RNR3*.

We next turned our attention to two other proteins implicated in cellular regulation by Asf1, namely Htz1 and Bdf1. Our studies suggest a role for the conserved Htz1 variant of H2A in Asf1-dependent steps in transcriptional regulation of *RNR3*. The findings consistent with this role are as follows. First, *ASF1* interacts genetically with *HTZ1* (Krogan et al., 2003; Fig. 4.7). Second, the rate of *RNR3* induction by DNA damage signals is misregulated in *htz1*Δ cells (Fig. 4.4, C). Third, Htz1 is enriched at *RNR3* under benign conditions (Fig. 4.2, B) and its crosslinking to *RNR3* declines in response to genotoxic stress (Fig. 4.5, A). Finally, physical association of Asf1 with Htz1 under conditions of Asf1 overexpression is regulated by DNA damage signals (Fig. 4.6, B). Collectively, our results are consistent with a model in which Htz1 functions in a pathway that regulates *RNR3* mRNA synthesis by limiting transcription during the early phase of the DNA damage response. These results reveal that both Asf1 and Htz1 associate dynamically with *RNR3* and contribute to its transcriptional regulation by genotoxic stress signals. A further implication of our work is that Asf1 may link the molecular events of H3/H4 tetramer metabolism with steps of chromatin remodeling involving the H2A/H2B dimer, Htz1, and the SWR1 complex. From a mechanistic viewpoint, the precise role of Htz1 in *RNR3* transcription is not known and it remains unclear how Asf1 and Htz1 collaborate in the regulation of *RNR3*. These are likely to be

complex problems to solve, considering the multitude of mechanisms and factors already known to contribute to transcriptional regulation of *RNR3* (Zhang and Reese, 2004b).

Our results also suggest a role for Bdf1 in the metabolism of Asf1 at *RNR3*. Because *BDF1* deletion caused a modest but reproducible decrease in Asf1 crosslinking to the 5' end of the coding region of *RNR3* (Fig. 4.3, A), we speculate that Bdf1 contributes to association of Asf1 with *RNR3* under normal conditions, but is not exclusively required for such. Supporting this idea is the observation that Bdf1 directly binds to Asf1 in vivo and in vitro (Chimura et al., 2002). Bromodomains can also bind to the K5/K12 acetylated tail of H4 (Jacobson et al., 2000; Ladurner et al., 2003) and K5/K12 acetylated H4 is enriched in soluble Asf1 complexes (Tyler et al., 1999; Emili et al., 2001; Chimura et al., 2002). Perhaps then Bdf1 and Asf1 contribute to a network of physical interactions at *RNR3* which includes their binding to H3/H4 acetylated nucleosomes.

Specific association of the Snf2-related ATPase Isw2 with *RNR3* also appears to affect crosslinking of Asf1 to *RNR3* (Fig. 4.8, B). There exist a number of functional similarities between Asf1 and Isw2. Both proteins may be recruited to specific genes by different targeting proteins (Goldmark et al., 2000; Kent et al., 2001; Gelbart et al., 2005). Isw2-dependent changes in chromatin structure at specific loci to which Isw2 crosslinks have been characterized (Kent et al., 2001), and we have observed Asf1-dependent changes in chromatin structure at *RNR3*, a gene with which Asf1 physically associates (Fig. 3.7, A). Furthermore, deletion of either *ASF1* or *ISW2* results in a reduction in H3 or H4 crosslinking to the promoter of *RNR3*, but not the coding region (Zhang and Reese, 2004a; Fig. 3.7, C). Therefore, it appears that the chromatin structures established by Isw2 or Asf1 may involve their physical association with target loci. It was proposed by Fazzio et al. (2001) that loss of Isw2 remodeling activity may result in more transcriptional repressor binding sites exposed at the *SUC2* gene. It could be that an increase in binding site exposure on the DNA for a nucleosome, repressor, or subunit of the transcriptional machinery accounts for the increased crosslinking of Asf1 observed in *isw2Δ* cells (Fig. 4.8, B). Previous evidence indicates that Isw2 is involved in repositioning nucleosomal arrays at sites in the DNA which are bound by regulatory factors (Goldmark et al., 2000; Kent et al., 2001); by extension we propose that

crosslinking of Asf1 to the *RNR3* promoter may be reduced by Isw2-dependent remodeling activity.

The effect of *ISW2* deletion on the level of Asf1 crosslinking to *RNR3* (Fig. 4.8, B) resembles the previously reported effect of *ISW2* deletion on crosslinking of the Crt1-Tup1-Ssn6 repressor complex to the *RNR3* promoter. Zhang and Reese (2004a) reported that *ISW2* deletion results in a significant increase in crosslinking of both Crt1 and Tup1 to the *RNR3* promoter. The overall similarity of the effect of *ISW2* deletion on crosslinking of Asf1, Crt1, and Tup1 to *RNR3* is consistent with a model in which Isw2 inhibits maximal crosslinking of these proteins to *RNR3*, perhaps through Isw2-dependent alterations of nucleosome positioning or due to steric hindrance.

ChIP experiments in yeast have demonstrated that Asf1 and Isw2 show a significant level of crosslinking to multiple loci (Kent et al., 2001; Alen et al., 2002; McConnell et al., 2004; Zhang and Reese, 2004a; Chapter 3). In *Drosophila*, both Asf1 and ISWI have abundant localization on polytene chromosomes (Tyler et al., 2001; Moshkin et al., 2002; Deuring et al., 2000). Taken together, this evidence suggests a widespread role for both Asf1 and ISWI proteins in regulation of chromatin structure. In fact, Asf1 has previously been implicated in chromatin remodeling based on its genetic and physical association with another chromatin remodeling complex, Brahma (SWI/SNF), in *Drosophila* (Moshkin et al., 2002). Although Asf1 does not coimmunoprecipitate with Isw2 (Fig. 4.9, C), it may be that Asf1 and Isw2 functionally interact at multiple loci to which both proteins are physically associated. For example, the combined activities of SWI/SNF and the Gcn5 HAT facilitate full nucleosome remodeling of the *RNR3* promoter. However, subunits of SWI/SNF including Snf2, Snf5, and Swi3 do not copurify with Gcn5 (Gavin et al., 2002). Similarly, the combined action of Asf1 and Isw2 during transcriptional regulation of target genes may underlie their functional interaction.

Early meiotic genes are repressed by Isw2 in a pathway parallel to the Sin3–Rpd3 HDAC complex upon recruitment by Ume6 protein (Goldmark et al., 2000). Kent et al (2001) reported that *ISW2* deletion results in changes in chromatin structure at several genes in vivo, however these are not always associated with changes in transcription levels under repressive conditions. Based on the requirement for Isw2 in transcriptional

regulation during *RNR3* derepression, it may be that Isw2-dependent chromatin changes are involved in transcriptional regulation of these genes under physiological induction conditions. It is also possible that transcriptional deregulation of DNA damage response genes in MMS-treated *isw2Δ* cells influences cellular survival. For example, deletion of *ISW2* could allow for hyperinduction of other damage response genes, thereby increasing the capacity for DNA repair.

Testing for the possibility of a genetic relationship revealed that Isw2 plays a negative role in survival of *asf1Δ* cells during MMS treatment and growth at 30°C. In the absence of Asf1, it appears that Isw2 interferes with an activity that enables the cell to survive DNA damage. How does Isw2 expression interfere with survival in *asf1Δ* cells during the response to DNA damage? One possibility is that Isw2 alters the ability of another chromatin assembly complex such as CAF-1 to assemble nucleosomes during DNA repair. For instance, it may be that Isw2 promotes or interferes with CAF-1 activity and this affects cellular survival. Alternatively, Isw2 could alter the chromatin state established and/or maintained by CAF-1, as Isw2-dependent nucleosome remodeling has been demonstrated at numerous loci (Kent et al., 2001; Ruiz et al., 2003; Zhang and Reese, 2004a). Although there clearly exists a functional relationship between Asf1 and Isw2 during genotoxic stress, it is evident from the incomplete suppression of *asf1Δ* MMS-sensitivity by loss of *ISW2* that other factors are involved. Previous results have suggested that the parallel functions of the Isw2 and Sin3-Rpd3 chromatin remodeling complexes make the phenotypes of *isw2Δ* mutants subtle (Fazzio et al., 2001; Goldmark et al., 2000), and this redundancy may contribute to the relatively modest but reproducible genetic interaction observed between Asf1 and Isw2. Multiple possibilities exist for the role(s) played by Isw2 that impact Asf1 function in an MMS-treated wild type cell. It could be that the DNA repair-coupled chromatin assembly function performed by Asf1 is inhibited by Isw2. For example, Isw2 may bind to and sequester a pool of Asf1 molecules under genotoxic stress conditions, thereby preventing Asf1 from performing its repair-induced functions. Alternatively, Isw2 may remodel nucleosomes following Asf1-dependent chromatin assembly at DNA repair sites in a manner that does not support recovery from damage and therefore impedes cellular survival. It will be

important to test these and other possibilities in the future in order to dissect the complex functional interplay between these chromatin regulators.

**Table 4.1.** Yeast strains used in Chapter 4

| Strain | Genotype |
|---|---|
| BY4741[a] | MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| BY4741 asf1Δ[a] | MATa *asf1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| BY4741 crt1Δ[a] | MATa *crt1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| BY4741 htz1Δ[a] | MATa *htz1Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| BY4741 isw2Δ[a] | MATa *isw2Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| Asf1-TAP[a] | BY4741, *HIS3::ASF1-TAP* |
| Htz1-TAP[a] | BY4741, *HIS3::HTZ1-TAP* |
| Bdf1-TAP[a] | BY4741, *HIS3::BDF1-TAP* |
| Isw2-TAP[a] | BY4741, *HIS3::ISW2-TAP* |
| YJW1 | BY4741 [pYEX-GST-ASF1][b] |
| YJW3 | BY4741 [pYEX-GST][b] |
| YJW6[c] | MATa *leu2 trp1 ura3-52 prc1-407 pep4-3 prb1-112 ASF1-4HA::TRP1* |
| YJW7[d] | MATa *ade2-1 ura3-1 his3-11,15 leu2-3, 112 trp1-1* |
| YJW34 | YJW6, *crt1Δ::kanMX6* |
| YJW71 | BY4741 *asf1Δ, HIS3::BDF1-TAP* |
| YJW72 | BY4741 *htz1Δ, asf1::HIS3* |
| YJW73 | BY4741 *asf1::HIS3* |
| YJW75 | YJW6, *yox1Δ::kanMX6* |
| YJW76 | YJW6, *htz1Δ::kanMX6* |
| YJW77 | YJW6, *dst1Δ::kanMX6* |
| YJW78 | YJW6, *spt4Δ::kanMX6* |
| YJW88 | BY4741 *asf1Δ*, HIS3::*HTZ1*-TAP |
| YJW89 | BY4741 *asf1Δ*, HIS3::*SWR1*-TAP |
| YJW91 | BY4741 *asf1Δ* [pYEX-GST][b] |
| YJW96 | YJW88 [pYEX-GST][b] |
| YJW97 | YJW89 [pYEX-GST][b] |
| YJW98 | YJW71 [pYEX-GST][b] |
| YJW102 | YJW88 [pYEX-GST-ASF1][b] |
| YJW103 | YJW89 [pYEX-GST-ASF1][b] |
| YJW104 | YJW71 [pYEX-GST-ASF1][b] |
| YJW105 | YJW6, *bdf1Δ::kanMX6* |
| YJW129 | YJW6, *isw2Δ::kanMX6* |
| YJW131 | BY4741 *isw2Δ, asf1::HIS* |
| YJW146 | BY4741 [pYEX-GST-ISW2][b] |
| YJW148 | BY4741 *asf1Δ* [pYEX-GST-ISW2][b] |
| YJW158 | Isw2-TAP, *asf1Δ::kanMX6* |
| UCC3612[e] | MAT*α lys2-801 trp1-63 hml::URA3 ade2-101 his3-200 leu2-1 ura3-52 asf1::HIS3* |

[a]Supplied by Open Biosystems.
[b]Plasmids from the collection of Martzen et al., 1999 (supplied by Research Genetics). Strains transformed with pYEX-GST-ASF1 expressed the correctly sized GST fusion protein and the plasmid fully complemented the growth and MMS-sensitivity phenotypes of *asf1Δ* cells. Strains transformed with pYEX-GST-ISW2 expressed the correctly sized GST fusion protein.
[c]Strain from Ann Ehrenhofer-Murray (Meijsing and Ehrenhofer-Murray, 2001).
[d]Strain from Rodney Rothstein (W303-1a; Thomas and Rothstein, 1989).
[e]Strain from Dan Gottschling (Singer et al., 1998). This PCR product of *asf1::HIS* was used to create YJW73 by homologous recombination and one-step disruption.

**Table 4.2.** Comparison between *asf1*Δ and *htz1*Δ microarray data

| YORF | matx_htz1_ S288C | DeRisi_Array_ htz_inverted1 | matx_htz1_ rpt | matx_htz1_ rpt_inverted | Avg Fold Δ in *asf1Δ* |
|---|---|---|---|---|---|
| YPR078C | 0.67 | 0.48 | 0.34 | 0.95 | A |
| YOR292C | 0.41 | 1.06 | 0.31 | 0.77 | 1.60 |
| YOR289W | 1.02 | 1.42 | 1.34 | 1.00 | NC |
| YOR220W | 0.65 | 0.58 | 0.74 | 0.44 | NC |
| YOR185C | 0.51 | 0.72 | 0.83 | 0.44 | 7.70 |
| YOR173W | 0.53 | 0.78 | 0.55 | 0.55 | 3.20 |
| YOR049C | 0.37 | 0.93 | 0.38 | 0.67 | 1.90 |
| YOR009W | 0.19 | 0.96 | 0.84 | 0.70 | 1.70 |
| YOL155C | 1.80 | 0.28 | 1.46 | 0.82 | 4.90 |
| YOL150C | 0.56 | 0.99 | 0.71 | 0.80 | 1.60 |
| YOL053C-A | 1.41 | 1.04 | 1.48 | 1.58 | 17.70 |
| YOL016C | 0.41 | 0.99 | 0.37 | 0.65 | -2.20 |
| YNR044W | 1.72 | 0.73 | 1.75 | 1.68 | -1.50 |
| YNL279W | 1.01 | 1.21 | 1.29 | 1.43 | 1.60 |
| YNL274C | 0.98 | 0.46 | 1.06 | 0.64 | 2.40 |
| YNL208W | 1.24 | 0.29 | 1.20 | 0.32 | 1.70 |
| YNL160W | 1.50 | 0.62 | 1.28 | 0.61 | 6.30 |
| YNL015W | 0.70 | 0.85 | 1.06 | 1.15 | 3.20 |
| YMR291W | 0.96 | 0.44 | 0.20 | 0.92 | 1.90 |
| YMR251W-A | 0.99 | 0.86 | 1.30 | 0.50 | NC |
| YMR250W | 0.89 | 1.45 | 0.90 | 0.92 | 4.40 |
| YMR244C-A | 0.31 | 0.85 | 0.72 | 0.58 | 1.90 |
| YMR232W | 0.83 | 0.90 | 0.58 | 1.08 | A |
| YMR169C | 0.68 | 1.18 | 0.06 | 0.65 | 3.90 |
| YMR107W | 0.65 | 1.32 | 0.65 | 0.66 | 13.00 |
| YMR105C | 1.67 | 1.82 | 0.63 | 1.54 | 5.10 |
| YMR090W | 0.69 | 0.67 | 0.99 | 0.75 | 2.70 |
| YML128C | 0.86 | 0.70 | 0.77 | 0.52 | 5.10 |
| YML100W | 0.99 | 0.46 | 0.75 | 1.18 | 2.50 |
| YML048W-A | 1.01 | 1.95 | 1.91 | 1.82 | 1.50 |
| YML047C | 1.34 | 1.30 | 1.90 | 2.22 | A |
| YLR438W | 1.32 | 0.87 | 1.33 | 0.71 | 1.80 |
| YLR346C | 0.26 | 1.22 | 1.15 | 0.98 | -3.80 |
| YLR327C | 1.55 | 1.48 | 1.71 | 1.32 | 6.10 |
| YLR297W | 0.48 | 0.82 | 0.41 | 0.54 | 3.00 |
| YLR258W | 0.88 | 1.49 | 0.16 | 0.97 | 3.20 |
| YLR226W | 0.63 | 0.44 | 1.16 | 0.29 | 1.90 |

| | | | | | |
|---|---|---|---|---|---|
| YLR186W | 0.88 | 0.59 | 0.93 | 0.65 | NC |
| YLR142W | 0.70 | 0.77 | 1.14 | 1.14 | 2.10 |
| YKR091W | 0.82 | 1.06 | 1.08 | 0.54 | 1.70 |
| YKR076W | 0.32 | 1.02 | 0.62 | 0.45 | 3.50 |
| YKR049C | 0.82 | 1.20 | 0.97 | 0.34 | 2.20 |
| YKL151C | 0.96 | 1.45 | 1.16 | 0.68 | 2.00 |
| YKL141W | 0.73 | 0.97 | 0.89 | 0.19 | 1.40 |
| YKL091C | 0.70 | 0.88 | 0.77 | 0.58 | 1.60 |
| YKL085W | 0.77 | 0.87 | 0.64 | 0.21 | 1.40 |
| YKL065C | 0.51 | 0.95 | 0.64 | 0.38 | NC |
| YKL026C | 0.37 | 0.80 | 0.67 | 0.94 | 2.50 |
| YJR150C | 1.12 | 1.25 | 1.82 | 2.65 | A |
| YJR004C | 1.44 | 1.27 | 1.39 | 1.43 | 1.70 |
| YJL211C | 0.65 | 0.52 | 0.70 | 0.42 | A |
| YJL210W | 0.41 | 0.58 | 0.54 | 0.64 | NC |
| YJL164C | 0.58 | 0.98 | 0.06 | 0.99 | 2.10 |
| YJL161W | 0.49 | 0.60 | 0.55 | 0.75 | 5.90 |
| YIL169C | 1.07 | 0.74 | 1.29 | 0.80 | A |
| YIL117C | 1.01 | 1.38 | 0.91 | 0.72 | 2.80 |
| YIL111W | 0.75 | 1.42 | 0.77 | 0.64 | 1.70 |
| YIL101C | 0.51 | 1.20 | 0.32 | 0.57 | 4.10 |
| YIL082W | 0.97 | 0.61 | 1.33 | 1.01 | A |
| YIL080W | 0.82 | 0.62 | 0.78 | 1.39 | NC |
| YHR087W | 1.66 | -0.03 | 1.27 | 1.59 | 5.10 |
| YHR059W | 0.38 | 1.06 | 1.05 | 0.34 | 1.70 |
| YHL021C | 1.06 | 1.08 | 0.55 | 0.48 | 1.50 |
| YGR248W | 0.87 | 1.21 | 0.67 | 0.86 | 4.90 |
| YGR244C | 0.57 | 1.26 | 0.37 | 0.58 | 3.00 |
| YGR161C | 1.29 | 1.58 | 0.97 | 0.91 | 2.10 |
| YGR142W | 0.77 | 1.23 | 0.25 | 0.33 | -2.10 |
| YGR088W | 0.95 | 0.23 | 0.83 | 0.73 | 4.30 |
| YGL121C | 0.77 | 0.51 | 1.00 | 1.04 | 8.90 |
| YGL090W | 1.11 | 0.48 | 1.04 | 0.84 | -2.10 |
| YGL089C | 1.33 | 1.26 | 1.50 | 0.56 | 1.70 |
| YGL037C | 0.86 | 0.95 | 0.70 | 0.92 | 1.60 |
| YGL032C | 0.72 | 0.36 | 1.69 | 0.90 | NC |
| YFR053C | 1.93 | 1.44 | 2.09 | 1.71 | 5.10 |
| YFR017C | 1.32 | 0.94 | 0.72 | 0.96 | 2.50 |
| YFR015C | 1.53 | 0.77 | 0.30 | 1.06 | 2.10 |
| YFL014W | 2.22 | 1.93 | 2.55 | 2.07 | 69.00 |
| YER079W | 0.70 | 0.86 | 0.62 | 0.62 | NC |
| YER067W | 1.56 | 1.70 | 1.55 | 1.04 | 1.90 |

| | | | | | |
|---|---|---|---|---|---|
| YER066C-A | 1.16 | 0.79 | 0.88 | 0.95 | NC |
| YER053C | 0.66 | 1.57 | 0.33 | 0.71 | 1.40 |
| YER037W | 0.52 | 0.77 | 0.50 | 0.57 | 3.00 |
| YEL024W | 0.58 | 0.70 | 0.68 | 0.49 | 3.10 |
| YDR453C | 0.28 | 1.02 | 0.41 | 1.04 | 1.60 |
| YDR391C | 0.67 | 1.00 | 0.63 | 0.46 | NC |
| YDR342C | 1.14 | 1.21 | 0.82 | 0.76 | 2.20 |
| YDR171W | 1.28 | 1.43 | 0.94 | 1.37 | NC |
| YDR070C | 0.95 | 2.13 | 0.93 | 1.47 | 9.80 |
| YDR055W | 0.60 | 1.01 | 0.67 | 0.40 | 1.40 |
| YDR032C | 0.71 | 0.70 | 0.70 | 0.56 | 1.7 |
| YDR003W | 0.32 | 1.01 | 0.43 | 0.64 | NC |
| YDL204W | 0.59 | 0.78 | 0.32 | 0.52 | 5.10 |
| YDL181W | 0.96 | 1.12 | 1.48 | 0.86 | 13.50 |
| YDL110C | 0.58 | 1.06 | 0.70 | 0.20 | 1.60 |
| YCL055W | 0.58 | 1.28 | 0.70 | 0.54 | NC |
| YCL040W | 1.06 | 0.37 | 0.67 | 1.40 | 2.50 |
| YCL027W | 0.71 | 0.53 | 0.55 | 1.09 | NC |
| YBR214W | 0.77 | 0.58 | 0.51 | 0.54 | 1.00 |
| YBR126C | 0.72 | 0.67 | 0.31 | 0.99 | 1.50 |
| YBR072W | 2.11 | 1.22 | 2.17 | 1.95 | 6.70 |
| YBR067C | 1.27 | 0.15 | 1.14 | 0.24 | NC |
| YBR066C | 0.82 | 0.72 | 0.59 | 0.65 | NC |
| YBL099W | 1.61 | 0.51 | 2.33 | 1.88 | NC |
| YBL064C | 0.81 | 0.72 | 0.99 | 0.49 | 3.70 |
| YBL016W | 0.50 | 0.45 | 0.75 | 0.60 | NC |

NC, fold change for *asf1Δ* was less than 1.4; A, the gene was absent from the *asf1Δ* dataset.
Htz1 microarray data is from Meneghini et al., 2003. *asf1Δ* microarray data is from Appendix Tables 7.1 and 7.4, and J. Williams and M. Schultz, unpublished data.

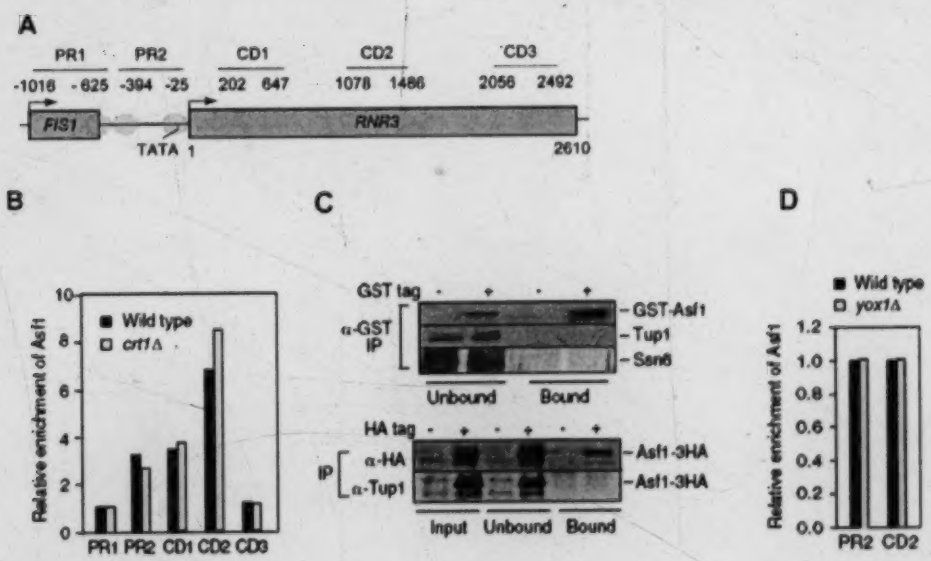


**Figure 4.1. Individual deletion of *CRT1* or *YOX1* does not affect Asf1 crosslinking to *RNR3*.** **A.** Location of primer sets used in ChIP experiments. **B.** Quantitative ChIP analysis of Asf1-4HA association with *RNR3* in wild type (YJW6) and *crt1Δ* (YJW34) cells. Normalization is to the signal for the PR1 primer set, which is assigned a value of 1. **C.** Upper panel. Anti-GST, anti-Tup1 and anti-Ssn6 immunoblots of fractions from strains expressing untagged (-) or GST-tagged (+) Asf1. Immunoprecipitation was performed with an anti-GST antibody. Lower panel. Anti-HA and immunoblots of fractions from strains expressing untagged (-) or HA-tagged (+) Asf1. Immunoprecipitation was performed with an anti-Tup1 antibody. **D.** Quantitative ChIP analysis Asf1-4HA association with *RNR3* in a *YOX1* deletion mutant. Occupancy by Asf1 in the deletion strain is expressed relative to recovery of Asf1-associated PCR products in the wild type strain (set to 1 for each primer set).

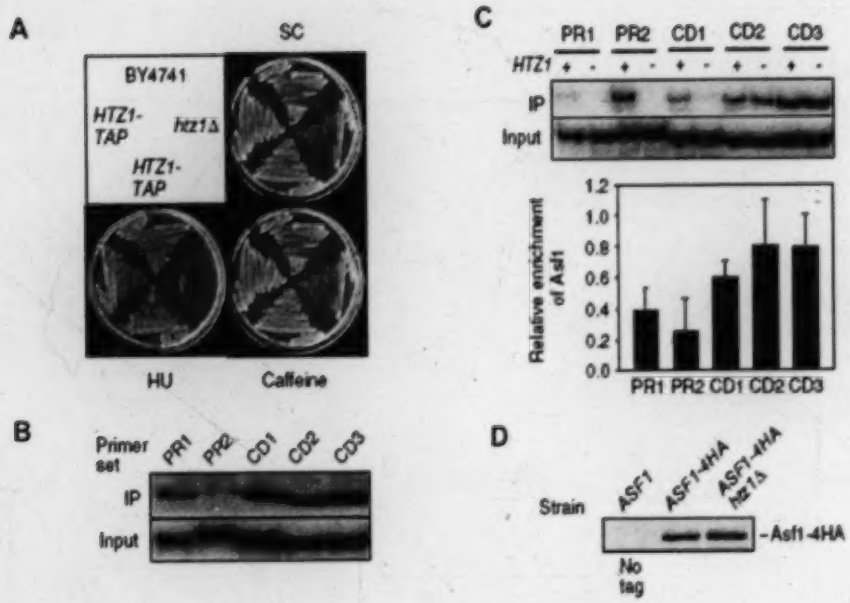


**Figure 4.2. Htz1 association with *RNR3* and effect of *HTZ1* deletion on Asf1 crosslinking to *RNR3*.** **A.** TAP tagging of Htz1 does not affect yeast cell growth in the presence of 150 mM hydroxyurea or 20 mM caffeine. Cells were plated on the indicated media and grown for 4 or 15 (caffeine) d at 30°C. Streaking of two independent colonies of the *HTZ1-TAP* strain is shown. **B.** Representative survey of Htz1-TAP crosslinking to *RNR3* by quantitative ChIP. **C.** Quantitative ChIP analysis of Asf1-4HA association with *RNR3* in wild type (YJW6) and *htz1Δ* (YJW76) cells. Fold enrichment of radiolabeled PCR products from *htz1Δ* was calculated relative to recovery of PCR products from the wild type strain (set to 1). The graph shows the mean of results from three independent experiments (+/- SD). Primer sets are the same as those indicated in Fig. 4.1, A. **D.** Bulk expression of HA-tagged Asf1 in wild type and *htz1Δ* cells. Immunoblotting was used to detect Asf1-4HA in whole cell lysates. No band co-migrating with Asf1-4HA is detected in lysate from cells which express untagged Asf1.

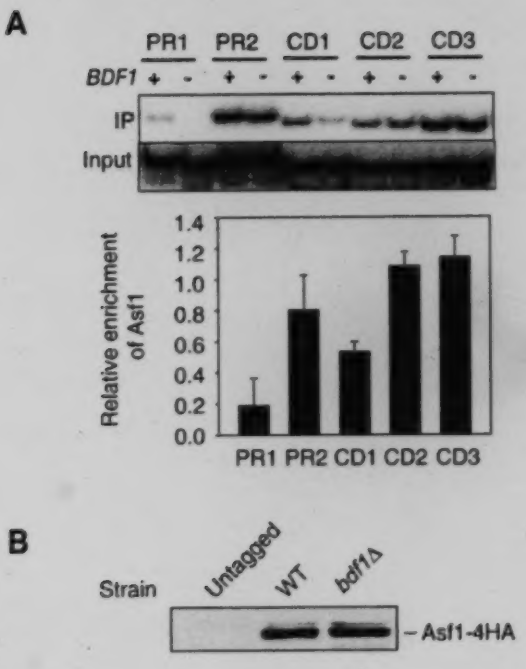


**Figure 4.3. The effect of *BDF1* deletion on Asf1 crosslinking to *RNR3*. A.** Quantitative ChIP analysis of Asf1-4HA association with *RNR3* in wild type (YJW6) and *bdf1Δ* (YJW105) cells. Fold enrichment of radiolabeled PCR products from the *bdf1Δ* strain was calculated relative to recovery of PCR products from the wild type strain (set to 1). The graph shows the mean of results from four independent experiments (+/- SD). **B.** Bulk expression of HA-tagged Asf1 in wild type and *bdf1Δ* cells. Immunoblotting was used to detect Asf1-4HA in whole cell lysates.

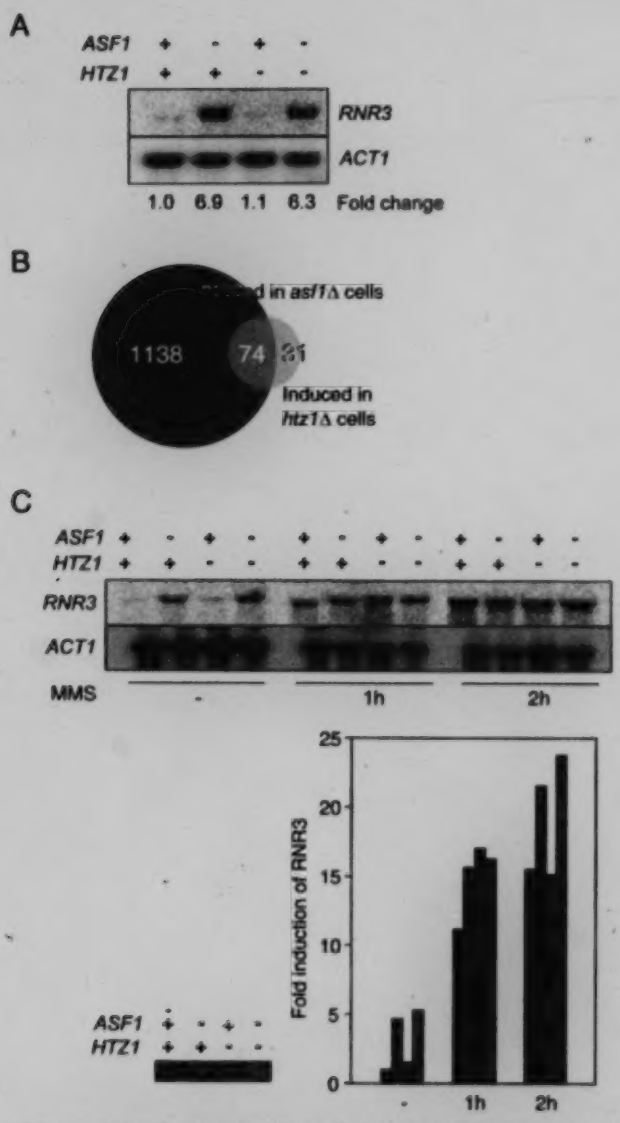


**Figure 4.4. Htz1 and the DNA damage response. A.** Northern blotting analysis of *RNR3* expression in wild type, *asf1Δ*, *htz1Δ* and *asf1Δhtz1Δ* (YJW72) cells under standard growth conditions. Expression relative to wild type (Fold change) was calculated by normalization to the signal for *ACT1*. **B.** Venn diagram of overlap between genes induced in *asf1Δ* cells (this study) and genes induced in *htz1Δ* cells (Meneghini et al., 2003). The data was filtered using a fold difference cutoff of 1.4, as adopted by Meneghini et al. (2003). **C.** Northern blotting analysis of *RNR3* expression in wild type, *asf1Δ*, *htz1Δ* and *asf1Δhtz1Δ* cells during treatment with 0.05% MMS. Expression relative to wild type was calculated by normalization to the signal for *ACT1*. The quantitative values presented represent the average result from 3 independent experiments; results between replicates varied by less than 10%.

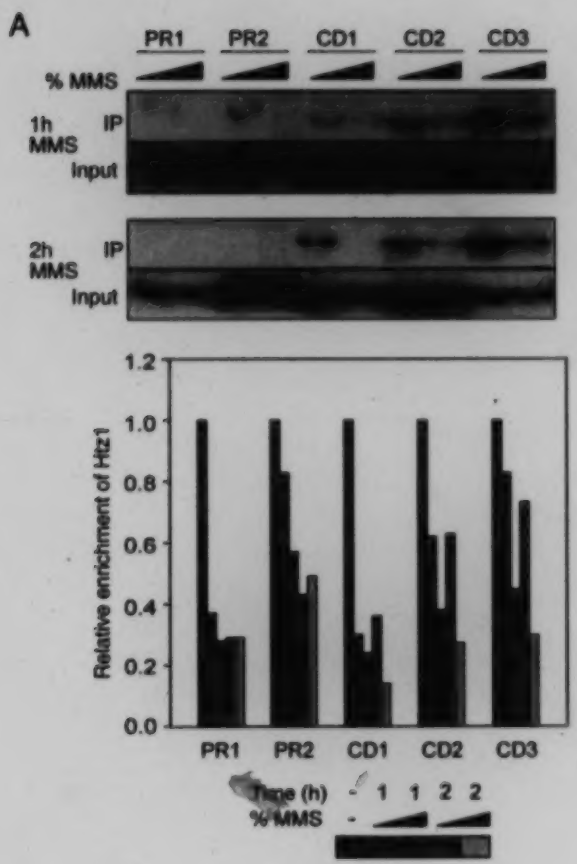


**Figure 4.5. Htz1 localization and the DNA damage response. A.** Quantitative ChIP analysis of Htz1-TAP association with *RNR3* after MMS treatment. Enrichment relative to recovery of individual PCR products from untreated cells (set to 1) was calculated. **B.** Bulk expression of TAP-tagged Asf1 in cells exposed to MMS. Immunoblotting was used to detect Htz1-TAP in whole cell lysates. No band co-migrating with Htz1-TAP is detected in lysate from cells which express untagged Htz1 (No tag).

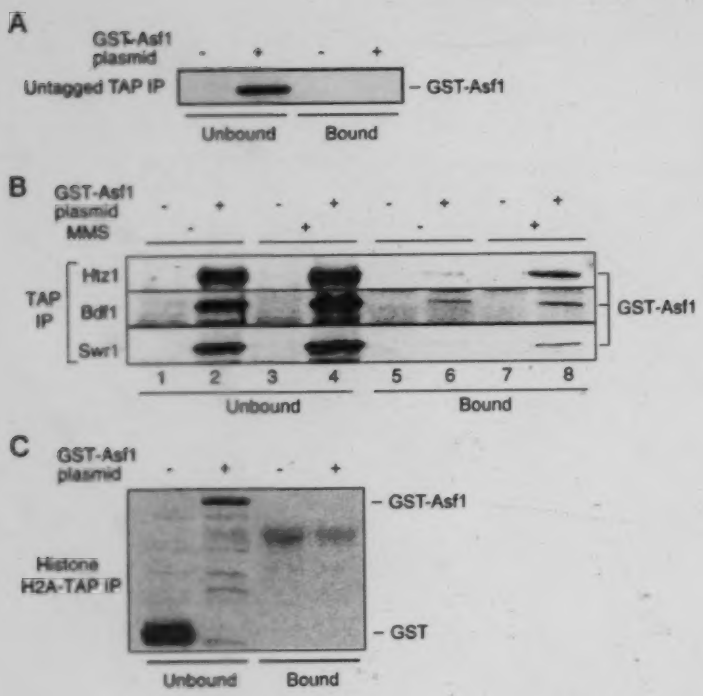


**Figure 4.6. Coimmunoprecipitation of Asf1 with Htz1 and Swr1. A.** Immunoblotting was used to detect GST-Asf1 in the unbound and bound fractions obtained by anti-TAP immunoprecipitation from lysates of strains expressing GST alone (-) or GST-Asf1 (+), but no TAP-tagged target protein. **B.** Immunoblotting was used to detect GST-Asf1 in the unbound and bound fractions obtained by anti-TAP immunoprecipitation from lysates of strains expressing TAP-tagged Htz1, Bdf1 or Swr1 as indicated at the left, in addition to GST alone (-) or GST-Asf1 (+). Treatment with 0.05% MMS was for 1 h. **C.** Immunoblotting was used to detect GST and GST-Asf1 in the unbound and bound fractions obtained by anti-TAP immunoprecipitation from the lysates of strains expressing TAP-tagged histone H2A in addition to GST alone (-) or GST-Asf1 (+).

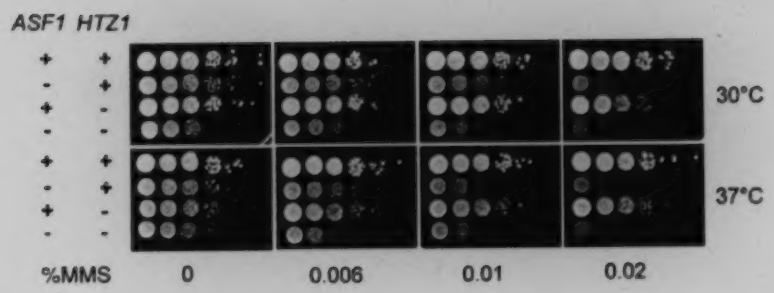


**Figure 4.7. Genetic interaction of *ASF1* with *HTZ1*.** Comparison of sensitivity to thermal stress and MMS was performed by plating serial dilutions of wild type, *asf1Δ*, *htz1Δ* and *asf1Δ htz1Δ* cells on rich medium at 30°C and 37°C in the presence or absence of MMS. Plates were photographed after 2d growth.

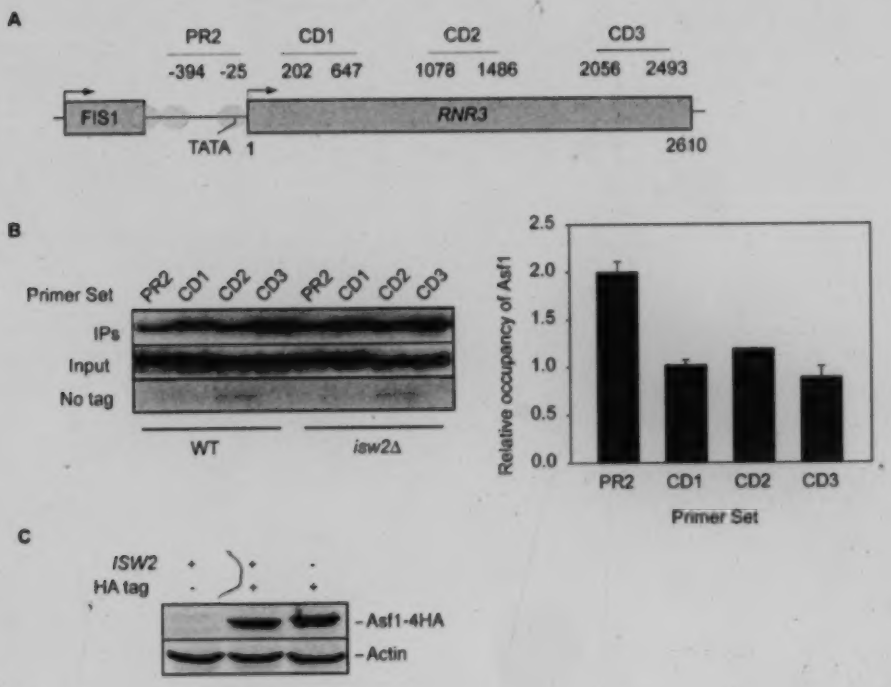


**Figure 4.8. The effect of *ISW2* deletion on Asf1 crosslinking to *RNR3*.** **A.** Location of primer sets used in ChIP experiments. **B.** Quantitative ChIP analysis of Asf1-4HA association with *RNR3* in wild type (YJW6) and *isw2Δ* (YJW129) cells. Fold enrichment of radiolabeled PCR products from *isw2Δ* samples was calculated relative to recovery of PCR products from the wild type strain (set to 1). The graph shows the mean of results from four independent experiments (+/- SE). **C.** Bulk expression of HA-tagged Asf1 in wild type and *isw2Δ* cells. Immunoblotting was used to detect Asf1-4HA in whole cell lysates. No band co-migrating with Asf1-4HA was detected in lysate from cells which express untagged Asf1. Equal protein loading of samples was confirmed by probing with a monoclonal antibody which recognizes actin. The experiment presented in panel C was performed by H. Mewhort.

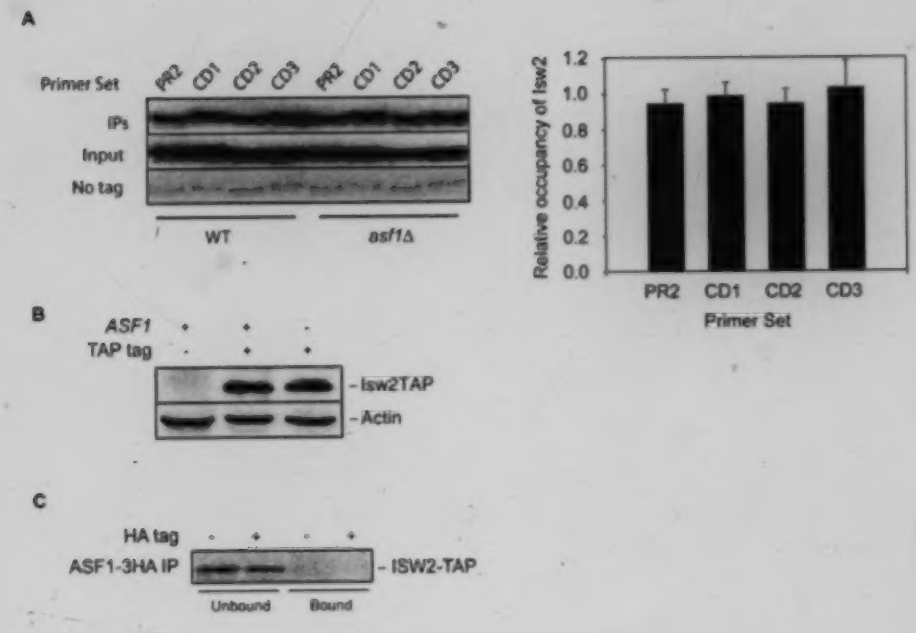


**Figure 4.9. The effect of *ASF1* deletion on Isw2 crosslinking to *RNR3*. A.** Quantitative ChIP analysis of Isw2-TAP association with *RNR3* in wild type and *asf1Δ* cells (YJW158). Fold enrichment of radiolabeled PCR products from *asf1Δ* cells was calculated relative to recovery of PCR products from the wild type strain (set to 1). The graph shows the mean of results from four independent experiments (+/- SE). **B.** Bulk expression of TAP-tagged Isw2 in wild type and *asf1Δ* cells. Immunoblotting was used to detect Isw2-TAP in whole cell lysates. No band co-migrating with Isw2-TAP is detected in lysate from cells which express untagged Isw2. Results from actin immunoblotting confirmed equal protein loading. **C.** Immunoblotting was used to detect Isw2-TAP in the unbound and bound fractions obtained by anti-HA immunoprecipitation from lysates of strains expressing untagged (-) or HA-tagged Asf1 (+). The experiment presented in panel B was performed by H. Mewhort.

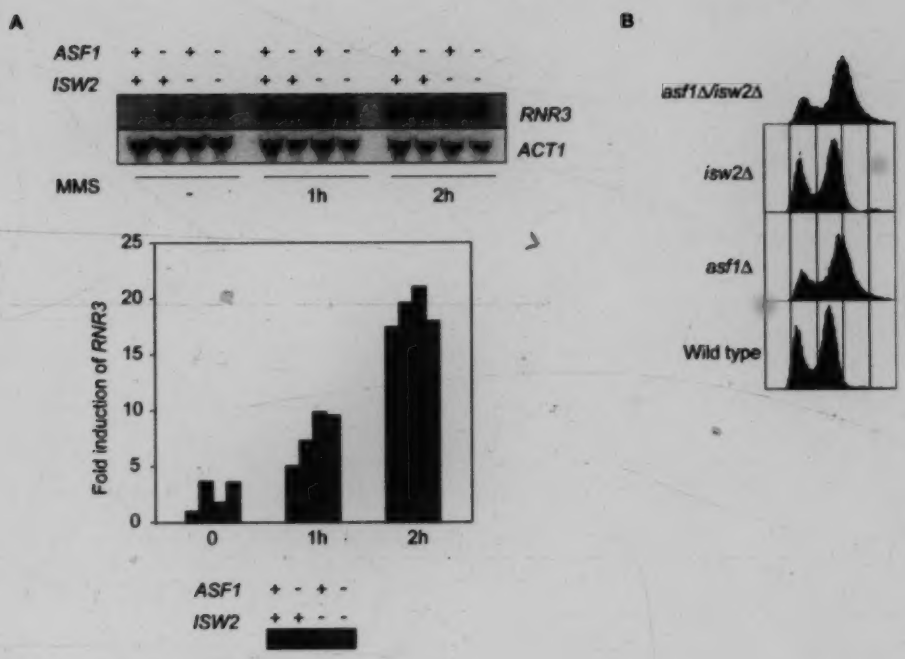


**Figure 4.10. The effect of *ISW2* deletion on *RNR3* transcription during the DNA damage response. A.** Northern blotting analysis of *RNR3* expression in wild type, *asf1Δ*, *isw2Δ* and *asf1Δisw2Δ* (YJW131) cells during treatment with 0.05% MMS. Expression relative to wild type was calculated by normalization to the signal for *ACT1*. The quantitative values presented represent the average result from 3 independent experiments; results between replicates varied by less than 10%. **B.** DNA content of cycling cells from the indicated strains was determined by flow cytometry analysis. Florescence intensity on the X axis is plotted against cell number on the Y axis.

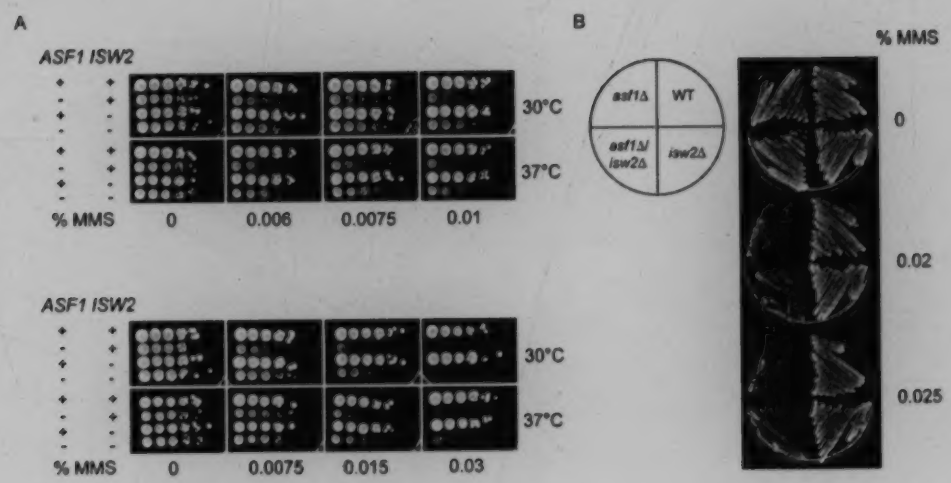


**Figure 4.11. The effect of *ISW2* deletion on *asf1Δ* growth in response to elevated temperature and DNA damage. A.** Comparison of sensitivity to thermal stress and MMS were performed by plating serial dilutions of two independent isolates from wild type, *asf1Δ*, *isw2Δ* and *asf1Δ isw2Δ* strains on rich medium at 30°C and 37°C in the presence or absence of MMS. Plates were photographed after 3 d growth. **B.** The streaking assay was used to compare the sensitivity of the indicated strains to intermediate MMS concentrations. Plates were grown at 30°C and photographed after 3 d growth.

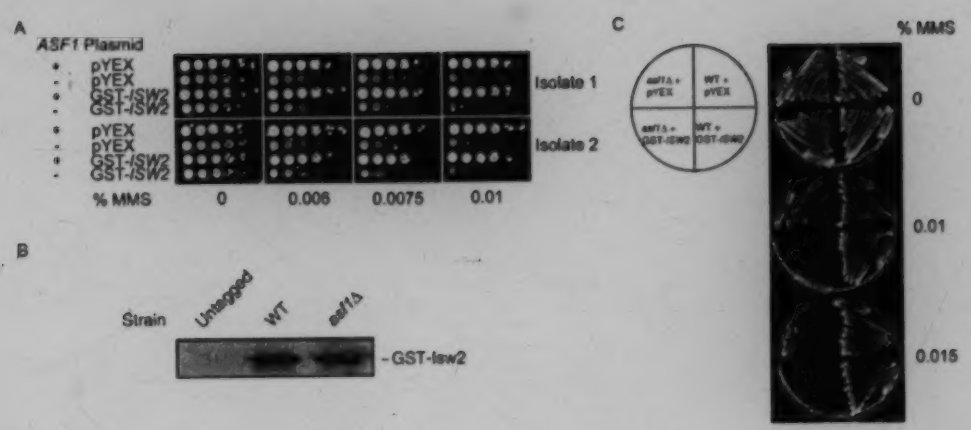


**Figure 4.12. The effect of GST-Isw2 overexpression on *asf1Δ* growth in response to DNA damage. A.** Comparison of sensitivity to MMS was performed by plating serial dilutions of two independent isolates from a wild type strain transformed with either vector (pYEX; YJW3) or GST-Isw2 high copy number plasmid (YJW146), or an *asf1Δ* strain transformed with either vector (YJW91) or GST-Isw2 high copy number plasmid (YJW148). Following copper induction of plasmid expression, plating was performed on selective medium at 30°C in the presence or absence of MMS. Plates were photographed after 4 d growth. **B.** Anti-GST immunoblots of total protein isolated from wild type or *asf1Δ* cells transformed with GST-Isw2. Expression levels of GST-Isw2 were monitored after copper induction. Protein extract isolated from a wild type strain expressing the vector was included as a negative control (Untagged). **C.** The streaking assay was used to compare the sensitivity of the indicated strains transformed with vector or GST-Isw2 to MMS. Plates were grown at 30°C and photographed after 4 d growth. The experiment presented in panel B was performed by H. Mewhort.

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**Part II:**

**Chapter 5:**

# Global control of histone modification by the Anaphase Promoting Complex

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**Summary:**

Acetylation and phosphorylation of the amino-terminal tails of the core histones fluctuate on a global scale in concert with other major events in chromosome metabolism. A ubiquitin ligase, the Anaphase Promoting Complex (APC), controls events in chromosome metabolism such as sister chromatid cohesion and may regulate H3 phosphorylation by targeting Aurora A, one of several S10-directed H3 kinases in vertebrate cells, for destruction by the proteasome. Our analysis of *apc10Δ* and *apc11*$^{ts}$ loss-of-function mutants reveals that the APC controls the global level of H3-S10 phosphorylation in cycling yeast cells. Surprisingly, it also regulates dephosphorylation of H3 and global deacetylation of H2B, H3 and H4 during exit from the cell cycle into $G_0$. Genetic, biochemical and microarray analysis suggests that APC-dependent cell cycle control of H3 phosphorylation is exerted at the level of an Aurora H3 kinase, Ipl1p, while APC-dependent transcriptional induction of *GLC7*, an essential H3 phosphatase, contributes to sustained H3 dephosphorylation upon cell cycle withdrawal. Collectively, our results establish that core histone acetylation state and H3 phosphorylation are physiologically regulated by the APC and suggest a model in which global reconfiguration of H3 phosphorylation state involves APC-dependent control of both an H3 kinase and a conserved phosphatase.

**Introduction:**

The flexible amino-terminal tails of the core histones are subject to a variety of site-specific post-translational modifications (reviewed in Fischle et al., 2003). Best characterized is acetylation, which occurs at conserved lysine residues in all core histones. Phosphorylation of H3 at S10 can be mechanistically linked to H3 acetylation and has also been well characterized (Hans and Dimitrov, 2001; Hsu et al., 2000; Lo et al., 2000, 2001). The global state of histone modification is subject to regulation by physiological cues through effects on histone-modifying enzymes (Carmen et al., 1999; Clarke et al., 1999; Howe et al., 2001; Reid et al., 2000; Rundlett et al., 1996; Vogelauer et al., 2000; Hsu et al., 2000). Typically the global reconfiguration of histone modification state under physiological circumstances coincides with other major events in chromatin metabolism. For example, S10 phosphorylation of H3 coincides with chromosome condensation during the cell cycle in many species (Hans and Dimitrov, 2001), as does deacetylation of H3 and H4 in mammals (Kruhlak et al., 2001). In yeast global H3 acetylation increases in S phase when the genome is being replicated (Krebs et al., 1999).

Considering the possibility that global reconfiguration of histone modification state might generally accompany global transitions in chromosome organization, we explored the regulation of histone acetylation and phosphorylation in another physiological context that involves genome-wide effects on chromosome structure and function, namely exit of yeast cells from proliferative growth into $G_0$ arrest. Nutrient deprivation triggers yeast cells to exit into $G_0$ from $G_1$ phase of the cell cycle (Werner-Washburne et al., 1993, 1996). Cells develop $G_0$ phenotypes through the orderly execution of a series of molecular events analogous to a differentiation program in higher eukaryotes (Herman, 2002; Werner-Washburne et al., 1993, 1996). These events include a reduction in proliferation rate, cell cycle arrest with a 1n content of DNA, large scale transcriptional reprogramming, and conformational changes in the chromosomes (DeRisi et al., 1997; Piñon, 1978; Piñon and Salts, 1977; Werner-Washburne et al., 1993).

Presumably the nature of the regulatory network that coordinates reconfiguration of histone modification state with other events in cell cycle exit includes upstream sensors of the environmental conditions that trigger entry into $G_0$, intermediate signaling

components, and effectors. Studies in metazoans have identified Aurora A as a cell cycle regulated H3-S10 kinase whose timely destruction is controlled by the APC (Crosio et al., 2002; Scrittori et al., 2001; Castro et al., 2002a, b; Honda et al., 2000; Littlepage and Ruderman, 2002). Although the physiological significance of H3 phosphorylation by Aurora A in metazoans is yet to be clarified, the above findings may be relevant to cell cycle control of the sole Aurora kinase in yeast, Ipl1p. Ipl1p is responsible for global H3-S10 phosphorylation during the cell cycle (Hsu et al., 2000). Because its bulk expression declines in $G_1$ when the APC is active, others have suggested that Ipl1p expression might be controlled by the APC in yeast (Biggins et al., 1999). Perhaps then an APC-dependent mechanism contributes to the observed repression of Ipl1p activity in $G_1$ (Buvelot et al., 2003). In this regard it is noteworthy that Ipl1p contains four RxxL motifs or putative destruction boxes (D boxes), which may serve as recognition motifs for ubiquitination by the APC (Glotzer et al., 1991; King et al., 1996).

Collectively, these data provisionally places the APC in a network controlling H3 phosphorylation during the cell cycle. Accordingly we considered the possibility that this ubiquitin ligase might also regulate histone modification state in response to nutrient signaling cues. Some additional information further encouraged this thinking. 1. The growth response of cycling yeast cells to glucose availability is controlled by the APC (Irniger et al., 2000). For example, a temperature sensitive mutation of an APC subunit involved in substrate recognition and/or enzymatic processivity (Apc10p/Doc1p; Carroll and Morgan, 2002; Passmore et al., 2003) is suppressed by culture in the absence of glucose (Irniger et al., 2000). Genetic evidence supports a model in which glucose-dependent signals inhibit APC functions related to the control of anaphase onset and mitotic exit (Irniger et al., 2000). In this model the APC is well placed to be a regulator of histone modification state during the $G_1$ to $G_0$ transition, especially given that glucose deprivation is a trigger for cell cycle withdrawal. 2. Our findings concerning the regulation of nucleosome assembly also relate to the idea that the APC might control histone modification state during entry into $G_0$. We previously discovered that mutation of the conserved *APC5* subunit of the APC inhibits replication-independent chromatin assembly in a yeast extract (Harkness et al., 2002). The reaction in this system is also sensitive to mutation of amino-terminal lysines in H4 that can be acetylated in vivo, and

H3/H4 from $G_2$/M cells are better substrates for assembly in the extract than are H3/H4 from S phase cells (Altheim and Schultz, 1999; Ling et al., 1996; Ma et al., 1998). It follows that a defect in histone metabolism may underlie the assembly defect of extracts from *apc5* mutant cells. The results presented in this chapter confirm the involvement of the APC in regulation of histone modification state during both the cell cycle and the transition from $G_1$ to $G_0$, as well as providing a mechanistic basis for APC-dependent regulation.

These general indications that the APC could be in a pathway controlling histone modification state in yeast are supported here by direct experimental observations. We find that the APC controls the S10 phosphorylation state of H3 during the cell cycle and in the course of cell cycle withdrawal following nutrient limitation. Whereas the APC likely controls H3 phosphorylation state during the cell cycle by effects on Ipl1p, an APC-dependent mechanism that controls $G_0$ transcription of the *GLC7* H3 phosphatase contributes significantly to reconfiguration of H3 phosphorylation state in $G_0$. Surprisingly, the APC also regulates global acetylation of H2B, H3 and H4 in response to nutrient signaling cues. Collectively, our results establish that distinct APC-dependent mechanisms regulate the global modification state of histones in yeast during active proliferation and during exit from the cell cycle into $G_0$.

**Results:**

**Changes in amino-terminal modification state of histones are associated with execution of the $G_0$ program**

The bulk expression and post-translational modification of histones H2B, H3 and H4 were examined during culture of BY4741 (wild type), in rich liquid medium. Growth curves were obtained by direct counting of cells with a hemacytometer (Fig. 5.1, A). We monitored viability by plating (not shown) and cell cycle arrest by flow cytometry (Fig. 5.1, B) to assess the suitability of strain BY4741 for studies of the response to nutrient withdrawal. We find that when proliferation has essentially stopped (60 hours, time point 7), approximately 95% of cells in a BY4741 culture have a 1n content of DNA and the budding index is 8%; after 1 week in culture all cells have a 1n content of DNA (Fig. 5.1, C). At time point 7 and later (3 and 14 days) cells have the same viability as at the time of seeding. We conclude that BY4741 is a suitable strain for analysis of events in chromatin metabolism associated with normal execution of the $G_0$ program of development.

In order to minimize histone proteolysis in this study, we TCA-precipitated total protein from cells that had been instantaneously solubilized in NaOH immediately after recovery by centrifugation. Because the total protein content of cells declines with growth rate (Fig. 5.2, A; see also Fuge et al., 1994), histone expression levels were determined on a per cell basis (in all figures we compare cell equivalents of lysate, where indicated 2X amount of lysate contains the protein from twice as many cells as 1X amount of lysate). We probed actin and the TATA binding protein (TBP) as loading controls; note that actin expression is uniform during the growth cycle and that, consistent with a previous report (Werner-Washburne et al., 1996), TBP is repressed as cells enter $G_0$ (Fig. 5.2, A).

Bulk histone expression was assessed by probing blots with antibodies raised against fusions of the amino-terminal tail of H2B, H3 and H4 with GST (Fig. 5.2, B), and an antibody that recognizes both unmodified and acetylated calf thymus H4. As shown in panel B, each histone is resolved as a single band in all samples. No slower migrating bands, which could be indicative of ubiquitination, were detected in our analysis of H2B. The bulk expression of H2B and H3 is essentially uniform throughout the growth cycle.

H4 expression increases during the first 8 hours of culture, then declines to a level at 18 hours (time point 4) that is maintained until 95% of cells are arrested with 1n DNA content (60 h, time point 7).

Acetylated histones were detected in the same samples used to monitor bulk histone expression. Blots were probed with separate antibodies that recognize the K9/K14 diacetylated isoform of H3, and K5/K8/K12/K16 tetra-acetylated H4 (and to a lesser extent acetylated H2B). Whereas the bulk expression level of H2B, H3 and H4 does not change as cells with 1n DNA content accumulate in a culture, amino-terminal lysine residues in H2B, H3 and H4 are progressively deacetylated (Fig. 5.2, C). This deacetylation starts after 18 hours and continues until the end-point of the experiment. We obtained an estimate of the fold change in H3 and H4 acetylation levels by quantitation of immunoblots of serially diluted samples from cells at early and late time points. The results were normalized to the recovery of unmodified histone. This analysis revealed that H3 and H4 acetylation levels decline approximately 2.3- and 1.6-fold respectively in the course of the transition from log phase growth (time point 3) to cessation of activate proliferation (time point 7).

Knowing that $G_0$ is entered from $G_1$ (Werner-Washburne et al., 1993, 1996) when H3 is dephosphorylated (Hsu et al., 2000), led us to suspect that H3 might shift toward the dephosphorylated state as a population of cells enters $G_0$. This possibility was examined in the experiment shown in Fig. 5.2, D. We indeed observe progressive dephosphorylation of H3 starting after 15 hours of culture (time point 3), slightly before histone deacetylation is initiated. Quantitation revealed that H3-S10 phosphorylation is 1.9-fold lower at time point 7 (60 h) than at time point 4 (18 h) in the growth cycle. Therefore execution of the $G_0$ program is accompanied at its onset by global changes in chromosome modification state due to decreased H2B/H3/H4 acetylation and H3-S10 phosphorylation. These findings suggest that the balance of activity between opposing histone-modifying enzymes is dynamic during the growth cycle. This analysis does not, however, provide any insight into the nature of the regulatory system that must couple cell cycle and nutrient signaling cues to biochemical events that directly underlie the reconfiguration of histone modification state.

**Developmentally programmed reconfiguration of histone modification state is perturbed in APC mutants**

A critical component of the regulatory system that establishes the histone modification state of early $G_0$ cells was identified in experiments that extended previous studies of nucleosome assembly. These experiments exploited a crude yeast extract in which nucleosome deposition, as measured by plasmid supercoiling, is sensitive to the acetylation state of endogenous H4 (Ma et al., 1998; Schultz, 1999). Based on our previous work suggesting that mutation of *APC5* is associated with defective supercoiling in vitro (Harkness et al., 2002), we assayed the supercoiling capacity of stationary phase extracts from mutants of three other well-characterized APC subunits (Harper et al., 2002). While relatively unaffected by deletion of either *APC9* or *CDC26* (Fig. 5.3, B), supercoiling activity was perturbed in extracts from a mutant lacking the Apc10p/Doc1p processivity/substrate recognition subunit of the APC (Fig. 5.3, A, B). This specific requirement for Apc10 and not the other APC subunits tested may reflect the direct regulatory function of Apc10 in APC activity through its recognition of substrates (Passmore et al., 2003). The supercoiling results suggest that, among other possibilities, defective chromatin assembly in vitro could be due to changes in the histone composition of extracts that stem from altered histone metabolism in vivo.

The analysis of *APC10* was extended by testing if its deletion impairs the developmental regulation of histone modification. Figure 5.2 shows a comparison of the state of histone modification in *apc10Δ* and wild type cells during the growth cycle. Deletion of *APC10* does not affect the bulk expression of H2B, H3 or H4 at any time in the growth cycle (Fig. 5.2, B). However at the point when the proliferation rate of wild type cells sharply declines (36 h, time point 6), H2B, H3, and H4 are more comprehensively deacetylated in *apc10Δ* than in wild type cells (Fig. 5.2, C). During further culture this difference becomes more pronounced, so that at 60 hours H3 and H4 are, respectively, 2.4 and 5-fold hypoacetylated in mutant compared to wild type cells. Phosphorylation of H3 at S10 is also misregulated in *apc10Δ* cells (Fig. 5.2, D). Surprisingly, H3 becomes hyperphosphorylated at the apparent point of entry into stationary phase (2.4-fold over wild type at time point 6), and then is slowly dephosphorylated as cells further accumulate in $G_0$. While dephosphorylation is

progressive, at the endpoint of this experiment H3 is still hyperphosphorylated at S10 in the mutant compared to wild type (wild type H3 phosphorylation level is 1.8-fold lower than in *apc10Δ* cells).

To confirm that aberrant histone acetylation and phosphorylation in *apc10Δ* cells reflects a defect in APC function, the regulation of these H3/H4 modifications was examined in a conditional mutant of *APC11*, the highly conserved RING-H2 subunit of the APC's ubiquitin ligase core (Gmachl et al., 2000; Harper et al., 2002; Leverson et al., 2000; Peters, 2002). Wild type and *apc11-13* (Leverson et al., 2000) cells were grown to early stationary phase at the permissive temperature (24˚C; Fig. 5.4, A), a condition that partially inactivates the APC in cycling *apc11-13* cells but does not affect viability (Leverson et al., 2000). Figure 5.4 shows the immunoblotting results for H3/H4 in *apc11-13* cells (panels C, D) and, for comparison, H4 in *apc10Δ* cells (panel B; in this instance bulk H4 was also detected by an antibody that recognizes all forms of calf thymus H4). Clearly the *apc10Δ* and *apc11-13* mutations confer similar histone acetylation phenotypes; bulk H3/H4 expression is not perturbed by the *apc11-13* mutation (Fig. 5.4, C), and both histones are aberrantly deacetylated in *apc11-13* cells (Fig. 5.4, D). Figure 5.4, D, further shows that S10 phosphorylation of H3 is elevated in *apc11-13* cells in $G_0$. Because histone acetylation and phosphorylation are perturbed when the catalytic function of the APC is compromised, it is likely that APC-dependent regulation of chromosome covalent modification state involves ubiquitination of a target protein(s).

**Aberrant reconfiguration of histone modification state in APC mutants is not an indirect consequence of lethal metabolic disruption**

Three cellular phenotypes are associated with the failure of *apc10Δ* cells to properly reconfigure the modification state of the histones. These phenotypes, however, do not account for abnormal histone metabolism during the growth cycle. The failure of *apc10Δ* cultures to reach the same stationary phase density as wild type cultures (Fig. 5.1, A) could account for abnormal histone metabolism in *apc10Δ* cells if the latter is generally associated with early cessation of proliferation in liquid culture. This possibility is ruled out by two observations: 1) other mutants that stop proliferating at a low density in liquid culture have the wild type pattern of H4 acetylation (*ubc1Δ* and

*spt20Δ*, V. Ramaswamy and M. Schultz, unpublished data), and 2) *apc11-13* cells have clear defects in histone metabolism (Fig. 5.4) but proliferate to the same density as wild type cells. Although wild type and *apc10Δ* strains are equally viable after 4 days in culture (Fig. 5.7, B), long-term survival is severely compromised in the mutant (M. Schultz, unpublished data). This observation raises the possibility that aberrant histone metabolism is caused by events that lead to inviability in $G_0$. We however exclude this possibility because *apc11-13* cells have the same histone modification phenotypes as *apc10Δ* cells but do not lose viability upon long-term culture.

As previously noted, normal progression into $G_0$ involves cell cycle exit to a state in which the nucleus has a 1n content of DNA. Thus 2n cells account for only ~5% of the total wild type population after 2.5 d in culture (Fig. 5.1, B, time point 7) and the budding index is 8%; after 1 week 2n cells have essentially disappeared (Fig. 5.1, C). In *apc10Δ* cultures, on the other hand, ~20% of cells have a 2n content of DNA after 2.5 d (Fig. 5.1, B) and the budding index is 18%. Furthermore, 2n *apc10Δ* cells in a growth-arrested culture are never recruited into the 1n population (Fig. 5.1, C). These observations raise the possibility that APC mutants are grossly defective for execution of the $G_0$ program, in which case a complex interplay of indirect effects and not an abnormal response to nutrient signaling cues could account for their histone modification phenotypes. We note, however, that in several important respects the growth cycle of *apc10Δ* cultures resembles that of wild type cultures. Cultures of both strains shift from containing about the same proportion of 1n and 2n cells during active proliferation to having a predominance of 1n cells when proliferation has ceased (Fig. 5.1, C), and total protein and TBP content decline with almost identical kinetics in wild type and *apc10Δ* cultures (Fig. 5.2, A). We conclude that the majority of cells in a growth-arrested *apc10Δ* culture have successfully executed at least some critical aspects of the normal developmental response to nutrient limitation. Although the 2n cells that persist in *apc10Δ* cultures may be in a $G_2$/M-like state, the experiments outlined below reveal that the histone modification phenotypes of *apc10Δ* cells are not due to $G_2$/M arrest.

**Cell cycle regulation of histone modification state in *apc10Δ* cells: disruption of H3-S10 phosphorylation but not H3/H4 acetylation**

Our flow cytometry analysis of stationary phase *apc10Δ* cultures reveals the presence of a subpopulation of 2n DNA cells. This result raises the possibility that when APC mutants are in $G_2$/M, histone metabolism is disrupted and it is this disruption that accounts for the histone modification profile of a $G_0$ population of *apc10Δ* cells. To explore this possibility, we examined H3/H4 acetylation and H3 phosphorylation states in wild type and *apc10Δ* cells uniformly arrested in $G_2$/M using nocodazole. As shown in Figure 5.5, A, $G_2$/M cells are equally represented in wild type and mutant cultures treated with nocodazole. Furthermore, there is no evidence of H3/H4 deacetylation or H3 hyperphosphorylation in *apc10Δ* cells (Fig. 5.5, B), in contrast to the situation in nutrient-limited cultures (Fig. 5.2, C, D). We conclude that *apc10Δ* cells that cease proliferation in response to nutrient limitation do not inherit a pattern of H3/H4 hypoacetylation or H3-S10 hyperphosphorylation that was established during $G_2$/M of the cell cycle.

We extended this analysis to characterization of the modification state of the histones in $G_1$ and S phase-arrested cells obtained by treatment with α-factor and hydroxyurea (HU) respectively. By FACScan analysis, *apc10Δ* and wild type cells respond identically to these treatments, with α-factor treatment yielding mostly $G_1$ cells, and HU treatment yielding a population in which most cells have initiated DNA replication (Fig. 5.6, A). Figure 5.6, B, shows that H3/H4 acetylation does not differ between wild type and mutant cells in either $G_1$ or S phase. In contrast, H3 is significantly hyperphosphorylated in *apc10Δ* cells arrested in $G_1$ (Fig. 5.6, B, C). This result suggests that Apc10p is required during the mitotic cycle for normal dephosphorylation of H3 in $G_1$. In summary, although one-fifth of stationary phase *apc10Δ* cells are in a $G_2$/M-like state, there is no defect in histone acetylation or phosphorylation during $G_2$/M when *apc10Δ* cells are actively proliferating. On the other hand, Apc10 is required for dephosphorylation of H3-S10 during $G_1$ in the mitotic cell cycle and during cell cycle exit into $G_0$.

**mRNA expression is globally disrupted in *apc10Δ* cells under conditions of nutrient limitation**

The extent to which the histone phenotype of *apc10Δ* cells in 2 d cultures (between time points 6 and 7) is associated with aberrant gene expression was tested

using microarray technology. Two independent experiments ($r^2 = 0.93$, n = 1249) were performed for each wild type versus *apc10Δ* comparison; fold change for each gene is the average of the two independent measurements (Appendix Tables 8.1 and 8.2). 15% (944) of the approximately 6,200 known and predicted protein coding genes are misregulated in mutant versus wild type cells (>/= 3-fold significance threshold). This effect is similar in scale to that observed when wild type cells enter the diauxic phase of the growth cycle (17% of genes affected; DeRisi et al., 1997). It is not as profound as the effect of depleting H3 and H4 (25% of present genes affected; Wyrick et al., 1999) and is in contrast to the modest response to deleting the *GCN5* HAT, which impinges on only 4% of genes (Lee et al., 2000). From a global perspective, the consequence of *APC10* deletion on gene expression is not specific to previously described chromosomal domains; *APC10*-responsive genes are distributed throughout the chromosomes (Fig. 5.7, A). Neither is the effect on expression limited to induction or repression; of the protein coding genes, 547 are repressed and 397 are induced. And because the net outcome in *apc10Δ* cells is repression of <3% of all genes (=140) at a stage when bulk acetylation is reduced by at least 50%, the acetylation phenotype is unlikely to simply represent the sum of chromatin remodeling events at individual genes that are misregulated in the mutant.

The pattern of transcriptional misregulation in *apc10Δ* cells is complex. The effects are not restricted to genes that normally change in activity during entry into $G_0$ (the diauxic shift; DeRisi et al., 1997). In fact, most genes in the *apc10Δ* gene set are not diauxic shift genes (66%; 620/924), and the fraction of diauxic shift genes in the mutant set (34%; 324/944) is similar to the fraction in the genome as a whole (~28%; 1740/6,200). The misregulated genes also do not substantially populate defined functional categories of gene product; at the 3-fold significance threshold, only one functional category (from PIR keyword categories in GeneSpring 4.1.5) is represented in the *apc10Δ* gene set (oxidative phosphorylation/membrane associated, $P = 0.024$). Nonetheless, some phenotypes related to nutrient utilization are possibly a direct consequence of transcriptional misregulation in the *apc10Δ* mutant. For example, two enzymes required for ethanol and glycerol utilization (Young et al., 2002) are strongly downregulated in *apc10Δ* cells (*ADH2*, 54-fold repression; *ACS1*, 8.7-fold repression),

which lose viability when grown on 2% glycerol or 3% glycerol plus 2% ethanol (Fig. 5.7, B).

Although the transcription phenotype of *apc10Δ* cells may represent a complex integration of direct effects of *APC10* deletion with indirect growth effects, it is informative to consider the results in terms of current ideas about the relationship between histone modification state and transcriptional regulation. For example, some changes in gene expression in *apc10Δ* cells (Fig. 5.7, C) are consistent with a dependence of repression on histone deacetylation (Knoepfler and Eisenman, 1999). In comparisons described here, the fold change of expression for genes that normally respond to glucose depletion is the maximum recorded during the diauxic shift (2-fold significance threshold; DeRisi et al., 1997). The behavior of many nuclear ribosomal (r) protein genes, normally downregulated during the diauxic shift by a mechanism involving HAT and HDAC complexes (Kurdistani et al., 2002; Rohde and Cardenas, 2003) is a typical example; 15 of 16 such genes in the *apc10Δ* gene set are hyper-repressed in the mutant. Conversely, the dampened induction of many $G_0$-activated genes (for example the 14 oxidative phosphorylation/membrane-associated genes in Fig. 5.7, C) suggests interference with acetylation events normally required for induction during the diauxic shift. Transcription phenotypes in *apc10Δ* cells however do not universally reflect the expected association of deacetylation with repression (Fig. 5.7, D). Of the 166 genes that are induced in *apc10Δ* cells and are responsive to nutrient limitation (DeRisi et al., 1997), 116 are normally repressed during the diauxic shift. Therefore, even in the context of global deacetylation some genes in *apc10Δ* cells are not appropriately repressed. Fifty genes that are over-expressed in *apc10Δ* cells are normally induced during the diauxic shift. Such $G_0$-activated genes therefore are hyperinduced in the *APC10* null background. While a number of mechanisms could account for this hyperinduction phenotype, it is possible that persistant S10 phosphorylation of H3 contributes to this pattern of misregulation in *apc10Δ* cells. This is consistent with evidence that H3-S10 phosphorylation promotes transcription at some promoters (Lo et al., 2001).

**Glc7p phosphatase is a potential effector in the APC-dependent pathway controlling H3 phosphorylation in $G_0$**

Microarray analysis revealed that in 2 d cultures mRNA expression of only one

histone-modifying enzyme is sensitive to *APC10* deletion. That gene is *GLC7*, the H3-S10 phosphatase of yeast. *GLC7* mRNA is 4.2-fold downregulated in *apc10Δ* cells in late post-diauxic phase/early $G_0$ (Fig. 5.7, D), whereas *IPL1* and *SNF1*, the H3-S10 kinases with which it interacts genetically (Hsu et al., 2000; Sanz et al., 2000), are unaffected. We used Northern blotting to confirm and extend this observation. As shown in Figure 5.8, A, *GLC7* expression is essentially identical in wild type and *apc10Δ* cells in early log phase (lanes 1, 2). On the other hand, consistent with the microarray result, *GLC7* is strongly repressed (7-fold) in early stationary phase *apc10Δ* cells (lanes 3, 4).

These results support a model in which normal stationary phase expression of *GLC7* is dampened when *APC10* is deleted. Consequently the H3-S10 kinase/phosphatase expression ratio is tipped in favor of the kinases and therefore hyperphosphorylation of H3 (in the simplest case because Glc7p downregulation causes H3 phosphatase activity to decline). This model predicts that the S10 phosphorylation state of H3 in $G_0$ is sensitive to Glc7p expression. In order to test this prediction we examined H3-S10 phosphorylation in a previously described *GLC7* mutant and its isogenic wild type partner. The *glc7-127* mutation evidently compromises the ability of the phosphatase to act on H3 in proliferating cells, even at the permissive temperature. Consequently, phospho-H3 accumulates in proliferating *glc7-127* cells (Hsu et al., 2000). As shown in Figure 5.8, B, H3 phosphorylation is also substantially elevated in *glc7-127* cells in early $G_0$ (there was no effect on histone acetylation). To rule out the possibility that H3 is hyperphosphorylated in *glc7-127* cultures in $G_0$ because they include more $G_1$-like (1n) cells than wild type cultures (see Fig. 5.6, A, B), the DNA content of cells in 3 d cultures was analyzed by flow cytometry. Figure 5.8, C, reveals that 1n cells are not more abundant in *glc7-127* than wild type cultures. We conclude that compromising Glc7p-dependent phosphatase activity does contribute to increased H3-S10 phosphorylation in $G_0$.

The results in Figures 5.7, D and 5.8, A, and the evidence that *GLC7* transcription is normally induced in nutrient-deprived cells (DeRisi et al., 1997; Feng et al., 1991), suggest that transcriptional regulation of *GLC7* contributes to developmental control of H3-S10 phosphorylation state during execution of the $G_0$ program. The effect of *APC10*

deletion on H3-S10 phosphorylation in $G_1$ (Fig. 5.6, B, C) raises the possibility that transcriptional regulation of *GLC7* by the APC also contributes to the control of H3 phosphorylation during the cell cycle. This hypothesis was explored by measuring *GLC7* expression in wild type and *apc10Δ* cells arrested in $G_1$ (Fig. 5.8, D). When Northern blotting results are quantitated after normalization to actin expression, it is clear that there is no difference in *GLC7* expression between $G_1$-arrested wild type and *apc10Δ* cells. Therefore the mechanism that accounts for H3-S10 hyperphosphorylation in *apc10Δ* cells likely involves an APC-dependent effect on *GLC7* transcription in $G_0$ but not in $G_1$.

*GLC7* and *APC10* are expected to interact genetically if they each contribute to control of H3 phosphorylation in $G_0$. To test this possibility we created *apc10Δ* and *apc10Δ glc7-127* mutants isogenic to the strains used in the experiments in Figure 5.8, B and C. Growth of these four strains was compared in plating assays, of which representative examples are shown in Figure 5.8, E (two independent isolates each of *apc10Δ* and *apc10Δ gl7-127* were used). Consistent with analysis in the BY4741 genetic background (Fig. 5.1, A), *APC10* disruption confers a slow growth phenotype (compare wild type to the two *apc10Δ* strains). Combination of the *apc10Δ* and *gl7-127* mutations has a synthetic effect in which the double mutant grows more slowly than either the *apc10Δ* or the *glc7-127* single mutant. This genetic interaction supports the hypothesis that *GLC7* and *APC10* both act to control the phosphorylation state of H3. Consistent with this interpretation, H3 hyperphosphorylation in $G_0$ is accentuated in the *glc7-127 apc10Δ* double mutant compared to its wild type and single mutant partners (Fig. 5.8, F).

**Ipl1p kinase is a potential effector in the APC-dependent pathway controlling H3-S10 phosphorylation during the cell cycle**

Results in the literature implicate Ipl1p in the control of H3 phosphorylation state in cycling cells, possibly as a component of a pathway involving the APC. Our genetic and biochemical analysis indeed supports the existence of an APC-dependent mechanism for regulation of Ipl1p, as outlined below. First, *IPL1* interacts genetically with *APC10*. High copy number vectors expressing Ipl1p tagged at the amino-terminus with the HA epitope or with GST were used to transform wild type and *apc10Δ* cells. As in Figure 5.8, E, cell growth was monitored by plating of two independent isolates of each

genotype and a vector control was included in the experiments with GST-Ipl1p. Figures 5.9, A and B show that overexpression of HA- or GST-tagged Ipl1p reproducibly impairs the growth of *apc10Δ* but not wild type cells. Second, expression of Ipl1p is misregulated in *apc10Δ* cells. Immunoblotting was used to monitor the steady state level of HA-Ipl1p in wild type and *apc10Δ* strains in Figure 5.9, A. In cells arrested in $G_1$ by treatment with α-factor, deletion of *apc10Δ* is associated with substantial accumulation of HA-Ipl1 (Fig. 5.9, C). Accumulation of previously identified APC substrates is a well-established phenotype of some APC mutants (Peters, 2002). We conclude that expression of Ipl1p is limited in $G_1$ by a mechanism requiring the APC. Because $G_0$ is entered from $G_1$ phase of the cell cycle, in APC mutants it is possible that elevated $G_1$ expression of Ipl1p persists in $G_0$ and contributes to S10 hyperphosphorylation of H3 in the context of nutrient limitation. This hypothesis is supported by the observed modest but reproducible elevation of H3-S10 phosphorylation which accompanies overexpression of HA-Ipl1p in $G_1$-arrested *apc10Δ* cells (Fig. 5.9, D).

**Discussion:**

The data presented in this chapter show that the global reconfiguration of histone modification state in yeast is regulated by APC-dependent mechanisms during the cell cycle and during the response to nutrient limitation. Because mutation of a regulatory subunit of the APC (*APC10*) and mutation of a subunit of its catalytic core (*APC11*) confer similar defects in the control of histone modification state, it is likely that the APC functions as a ubiquitin ligase to modulate histone phosphorylation and acetylation. If this is true and APC-targeted proteins that control histone modification state are regulated by proteolysis, then crippling the proteasome should confer histone phenotypes similar to those observed for *apc10Δ* and *apc11-13* cells. Analysis of H4 acetylation in a conditional mutant of an essential subunit of the 20 S proteolytic core complex of the proteasome (*PRE1*; Baumeister et al., 1998) bears out this prediction (V. Ramaswamy and M. Schultz, unpublished data). Published information about the physiological regulation of APC activity is consistent with the existence of an APC-dependent pathway controlling histone modification state. 1. The APC is functional during $G_1$ (Harper et al., 2002; Peters, 2002; Zachariae and Nasmyth, 1999) when H3 is dephosphorylated by an APC-dependent mechanism. 2. The APC in yeast is activated by glucose limitation (Irniger et al., 2000), the best-characterized trigger of entry into $G_0$. 3. A highly active form of the APC can be isolated from $G_0$ (terminally differentiated) mammalian cells (Gieffers et al., 1999; Peters, 2002). Because $G_0$ is normally entered from $G_1$ (Herman, 2002; Werner-Washburne et al., 1993, 1996), when the APC is active, the APC is evidently well-positioned to modulate histone modification during cell cycle exit.

**Global regulation of H3 phosphorylation state by mechanisms involving the APC**

H3 phosphorylation state fluctuates during the cell cycle in yeast by a mechanism that may involve regulation of the H3-S10 kinase, Ipl1p (Honda et al., 2000). Ipl1p is downregulated in $G_1$ compared to S-phase cells (Biggins et al., 1999), Ipl1p-dependent kinase activity declines as cells enter $G_1$ (Buvelot et al., 2003), and Ipl1p accumulates in $G_1$-arrested *apc10Δ* cells (Fig. 5.9, C). Collectively this evidence is consistent with the straightforward proposal, originally elaborated by Biggins et al. (1999), that bulk expression of Ipl1p during the cell cycle is controlled by the APC. This regulatory

mechanism contributes to cell cycle control of Aurora A-type H3-S10-kinases in higher organisms. Therefore an APC-dependent pathway for cell cycle regulation of this family of kinases appears to be conserved in eukaryotes. In this regard, the putative D box motifs found in Ipl1p likely play a crucial role in its timely destruction. Apc10 itself has recently been demonstrated to contribute to recognition of a D box sequence found in an APC substrate, and mutation of this motif caused a reduction in the processivity of the ubiquitination reaction (Carroll et al., 2005). This further strengthens the possibility that Apc10 itself directly contributes to regulation of Ipl1 protein levels.

The information at hand indicates that APC-dependent regulation of an H3 phosphatase in yeast contributes to physiological regulation of H3-S10 phosphorylation state during the growth cycle. Specifically, because bulk Glc7p-dependent PP1 activity increases as cells enter quiescence (Nigavekar et al., 2002) and is required for H3 dephosphorylation in $G_0$ (Fig. 5.8), we propose that $G_0$-induction of H3-S10 phosphatase activity is important for H3 dephosphorylation when nutrients become limiting (Fig. 5.2). How might the APC contribute to $G_0$-induction of H3 phosphatase activity? It is possible that the APC regulates expression of a Glc7 regulatory subunit. Another attractive possibility is based on the finding that $G_0$ expression of *GLC7* mRNA is repressed in *apc10*Δ cells (Fig. 5.7, 5.8). Thus we suggest that high level expression of Glc7p in $G_0$ is maintained, at least in part, by an APC-dependent mechanism that actively sustains *GLC7* mRNA synthesis. High-level expression of Glc7p is then permissive for induction of Glc7p-dependent PP1 activity (Nigavekar et al., 2002) and dephosphorylation of H3.

Consistent with our model that a nutrient-responsive pathway controls *GLC7* mRNA expression in $G_0$, *GLC7* has many features in common with genes known to be upregulated when nutrients are limiting. Northern blotting and microarray experiments have shown that *GLC7* is induced in $G_0$ (DeRisi et al., 1997; Feng et al., 1991) and its expression is sensitive to disruption of signaling by the target of rapamycin (TOR) kinases, which are required for execution of the $G_0$ program (Hardwick et al., 1999; Schmelzle and Hall, 2000). Furthermore, the *GLC7* promoter contains two optimally-positioned copies of the 'Stress Response Element' (STRE; CCCCT) commonly required for activation of $G_0$-induced genes (Lenssen et al., 2002; Moskvina et al., 1998). More generally speaking, the idea that an APC-dependent mechanism controls *GLC7*

transcription is consistent with recent evidence that the APC regulates expression of mammalian transcription factors SnoN (Stroschein et al., 2001; Wan et al., 2001) and HOXC10 (Gabellini et al., 2003).

In the simplest model (above), the APC contributes to maintenance of H3 dephosphorylation in quiescent yeast cells by controlling Glc7p-dependent H3 phosphatase activity. However, in other organisms phosphatases have been implicated in cell cycle control of H3 phosphorylation by virtue of their effects on H3 kinases. Most notably, fission yeast Aurora A and vertebrate Auroras A and B are inhibited by dephosphorylation, and the vertebrate Auroras are physically associated with phosphatases (Murnion et al., 2001; Sugiyama et al., 2002). It follows that budding yeast Glc7p may regulate the H3 phosphorylation state in $G_0$ by controlling H3 kinase activity. Accordingly, it will be interesting in the future to test whether Glc7p directly regulates Ipl1p in a pathway that includes the APC. Although cell cycle control of vertebrate and fission yeast Auroras by phosphatases has not been demonstrated, it may also be profitable, considering our results, to test whether any Aurora-directed phosphatase from these organisms is regulated by the APC.

**The APC integrates cell cycle progression and cell cycle exit signals that control distinct effector mechanisms of H3 dephosphorylation**

Budding yeast cells enter $G_0$ from $G_1$ of the mitotic cell cycle (Herman, 2002; Werner-Washburne et al., 1993, 1996). Therefore cells enter $G_0$ at a time in the cell cycle when the APC is active and an APC-dependent mechanism, most likely targeted degradation of Ipl1p, promotes H3 dephosphorylation. APC-dependent transcriptional induction of *GLC7* subsequently contributes to sustained dephosphorylation of H3 in $G_0$. This regulatory scheme has three interesting features. First, physiological regulation of H3 phosphorylation state is not restricted to APC-dependent mechanisms that impinge on the H3 kinase. Rather, both the H3 kinase and a phosphatase are regulated. Second, the regulatory mechanisms that contribute to global reconfiguration of H3 phosphorylation state differ according to the physiological circumstance. Fluctuation of kinase activity (Buvelot et al., 2003) in the context of constitutive phosphatase expression suffices to control H3 phosphorylation during the cell cycle. An additional mechanism, induction of the phosphatase, is engaged in response to nutrient limitation. While these mechanisms

are distinct, they are tightly coupled in the context of $G_0$ development: establishment of the hypophosphorylated state in $G_1$ by regulation of the kinase is reinforced in $G_0$ by regulation of the phosphatase. Third, the distinct mechanisms for controlling the H3 kinase:phosphatase ratio involves the same master regulator, the APC. Because it controls $G_1$ dephosphorylation of H3, during every cell cycle the APC promotes a wave of chromosome modification that will be reinforced by APC-dependent induction of Glc7p phosphatase if nutrients become limiting. From a broad perspective, control of histone modification state by the APC is reminiscent of its regulatory function during active proliferation, when it controls sequential cell cycle transitions by distinct mechanisms (Harper et al., 2002; Peters, 2002; Zachariae and Nasmyth, 1999).

**Regulation of histone acetylation by the APC**

Rpd3p is the HDAC subunit of a conserved transcriptional repressor complex (Alland et al., 2002). In cycling yeast cells Rpd3p participates in global histone deacetylation, perhaps by a mechanism involving binding of the Rpd3p complex to histones or histone-interacting proteins (Kurdistani et al., 2002; Vogelauer et al., 2000). H3 and H4 are not deacetylated when yeast *rpd3Δ* cells enter $G_0$, and therefore Rpd3p is also required for global deacetylation of H3/H4 in cells that have ceased proliferation (Sandmeier et al., 2002). Given that Rpd3p is involved in deacetylation of H2B (Suka et al., 2001), this enzyme may also contribute to global deacetylation of H2B reported here (Fig. 5.2, C). Relatively little is known about the regulation of Rpd3p. For example, it is not known if the intrinsic enzymatic activity of Rpd3p, its bulk expression, or its ability to globally bind to histones or histone-interacting proteins, increases during cell cycle withdrawal. *RPD3* mRNA is only modestly induced during the growth cycle (if at all; DeRisi et al., 1997) and its expression is not sensitive to deletion of *APC10* (Appendix Tables 8.1 and 8.2). Accordingly, global histone deacetylation in $G_0$ must not involve APC-dependent transcriptional induction of *RPD3*, but rather effects at the protein level (if indeed changes in Rpd3p expression or properties contribute to global reconfiguration of histone acetylation). If APC-dependent regulation of HAT activity plays a role in setting the level of global acetylation in $G_0$ then multiple HATs must be affected because neither Gcn5p nor Esa1p, whose mutation globally affects histone modification state in cycling cells, can acetylate both H3 and H4 (Reid et al., 2000; Suka et al., 2001). It is

also possible that APC-dependent changes in H3-S10 phosphorylation state globally influence H3-K9 acetylation (Edmondson et al., 2002; Lo et al., 2000, 2001).

**A possible cause-effect relationship between chromatin assembly and APC-dependent reconfiguration of histone modification state**

Because some APC mutants have a chromatin assembly defect, it is possible that histone replacement (Ahmad and Henikoff, 2002) contributes to the global reconfiguration of histone modification state in $G_0$. For example, perhaps the APC activates a chromatin assembly pathway that preferentially deposits acetylated histones. When this pathway is inactivated under conditions of nutrient limitation, or by mutation of the APC, other assembly pathways that preferentially use less modified histones could predominate. Against a background of degradation of free hyperacetylated histones, this mechanism would drive global replacement of hyper- with hypoacetylated histones. This idea is consistent with our evidence that chromatin assembly in vitro is responsive to the state of histone modification (Altheim and Schultz, 1999; Ma et al., 1998) and that extracts from *APC10* (Fig. 5.3) and *APC5* (Harkness et al., 2002) mutants have a defect in assembly activity. In order to critically test this possibility, it will be necessary to identify the assembly factors that are active in the crude yeast assembly system and test their use of modified versus unmodified histones during active proliferation and in $G_0$.

**Functional significance of global regulation of histone modification state**

Why is H3 phosphorylated during the cell cycle? Why in cycling cells is the global level of histone acetylation set to the observed level? Despite intense study in recent years, these questions await definitive answers (see for example, Hans and Dimitrov, 2001; Kristjuhan et al., 2002; Peterson, 2001). Accordingly, a comprehensive account of the functional significance of the changes in histone modification state that occur upon cell cycle exit in response to nutrient limitation is currently beyond our grasp. Nonetheless, at least in the case of the ribosomal protein genes, it seems highly likely that APC-dependent histone deacetylation contributes directly to transcriptional repression in $G_0$. Thus, histone deacetylation by a mechanism involving the Rpd3p HDAC and Esa1p HAT complexes is a critical step in repression of ribosomal protein genes in response to nutrient limitation (Kurdistani et al., 2002; Rohde and Cardenas, 2003) and we find that global deacetylation is associated with their hyper-repression in *apc10Δ* cells (Fig. 5.7,

C). On a more speculative note, an intriguing possibility regarding the global organization of chromosomes is worthy of consideration. There is evidence from sedimentation velocity analysis of chromosome complexes that the overall structure of chromosomes changes when cells enter $G_0$ (Piñon, 1978; Piñon and Salts, 1977). Perhaps this structural transformation involves genome-wide reconfiguration of the chemical modification state of the chromosomes by the APC-dependent mechanisms defined here. By extension, the observed level of global histone modification in cycling cells may help to establish a specific overall structure of chromosomes that is permissive for global events in chromosome metabolism that occur in cycling but not quiescent cells. Such events could include replication, sister chromatid cohesion and separation, and high overall transcriptional activity (in proliferating yeast cells transcription occurs throughout the chromosomes and involves 76% of all genes; Velculescu et al., 1997).

**Table 5.1.** Yeast strains used in Chapter 5

| Strain | Genotype |
|---|---|
| BY4741[a] | MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| BY4741 apc9Δ[a] | MATa *apc9Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| BY4741 apc10Δ[a] | MATa *apc10Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| BY4741 cdc26Δ[a] | MATa *cdc26Δ::kanMX6 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* |
| YAP160[b] | MATa *ura3-52 lys-801 ade2-101 trp1-Δ63 his3-Δ200 apc11:HIS3 leu2-Δ1::APC11;LEU2* |
| YAP160 apc11-13[b] | MATa *ura3-52 lys-801 ade2-101 trp1-Δ63 his3-Δ200 apc11:HIS3 leu2-Δ1::apc11-13;LEU2* |
| KT1112[c] | MATa *leu2 ura3 his3* |
| KT1640[c] | MATa *leu2 ura3 his3 glc7-127* |
| YJW52 | BY4741 [pCC1128-HA-IPL1][d] isolate 1 |
| YJW53 | BY4741 [pCC1128-HA-IPL1][d] isolate 2 |
| YJW54 | BY4741 *apc10Δ*[pCC1128-HA-IPL1][d] isolate 1 |
| YJW55 | BY4741 *apc10Δ*[pCC1128-HA-IPL1][d] isolate 2 |
| YJW62 | KT1112 *apc10Δ::kanMX6* isolate 3 |
| YJW63 | KT1112 *apc10Δ::kanMX6* isolate 4 |
| YJW64 | KT1640 *apc10Δ::kanMX6* isolate 1 |
| YJW65 | KT1640 *apc10Δ::kanMX6* isolate 4 |
| YJW152 | BY4741 [pYEX-GST][e] |
| YJW153 | BY4741 *apc10Δ*[pYEX-GST][e] |
| YJW154 | BY4741 [pYEX-GST-IPL1][e] isolate 1 |
| YJW155 | BY4741 [pYEX-GST-IPL1][e] isolate 2 |
| YJW156 | BY4741 *apc10Δ*[pYEX-GST-IPL1][e] isolate 1 |
| YJW157 | BY4741 *apc10Δ*[pYEX-GST-IPL1][e] isolate 2 |

[a]Supplied by Open Biosystems.
[b]Strains from Andrew Page (Leverson et al., 2000).
[c]Strains from Kelly Tatchell (Hsu et al., 2000).
[d]Plasmid from Clarence Chan (Kang et al., 2001; Kim et al., 1999).
[e]Plasmids from the collection of Martzen et al., 1999 (supplied by Research Genetics).

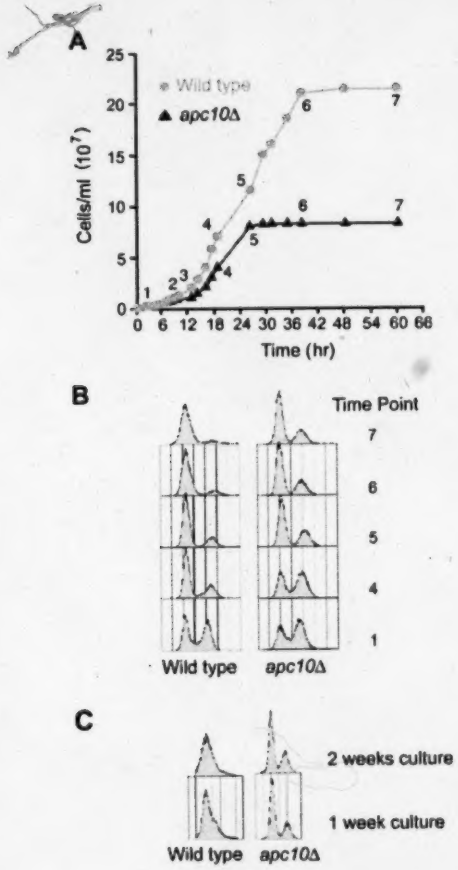


**Figure 5.1. Growth properties of wild type and *apc10Δ* cells in liquid culture. A.** Growth curves of wild type and *apc10Δ* strains in rich medium. **B.** Flow cytometry profiles of wild type and *apc10Δ* cultures at the time points in panel A. **C.** Flow cytometry profiles of wild type and *apc10Δ* cultures after 1 and 2 weeks. Florescence intensity on the X axis is plotted against cell number on the Y axis. The experiments presented in this figure were performed by V. Ramaswamy and J. Williams.

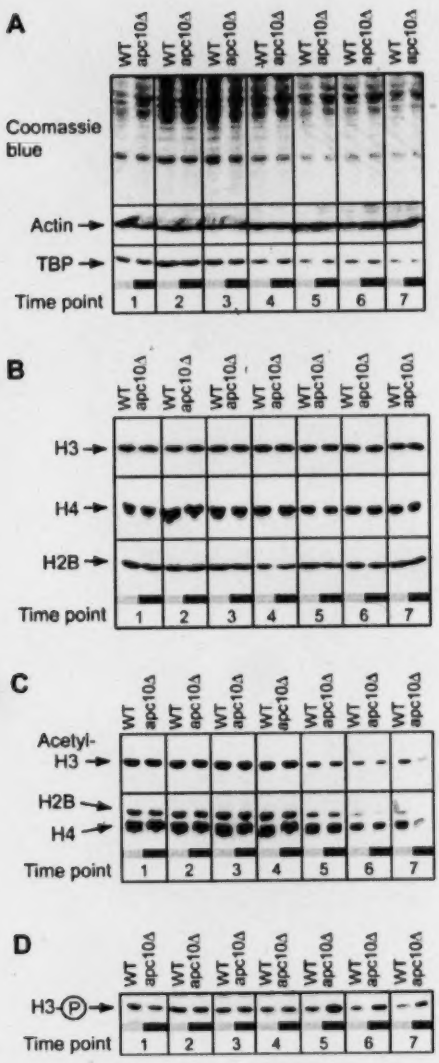


**Figure 5.2. Developmental regulation of histone modification state in wild type and *apc10Δ* cells. A.** Total protein content (Coomassie blue staining) and expression of actin and TBP in wild type (WT) and *apc10Δ* cells. **B.** Immunoblotting analysis of bulk H2B, H3, and H4 expression. **C.** Acetylation state of H2B, H3, and H4 as determined by immunoblotting. **D.** S10 phosphorylation state of H3 as determined by immunoblotting. All time points are as in Figure 5.1, A. The experiments presented in this figure were performed by V. Ramaswamy and reproduced by J. Williams.

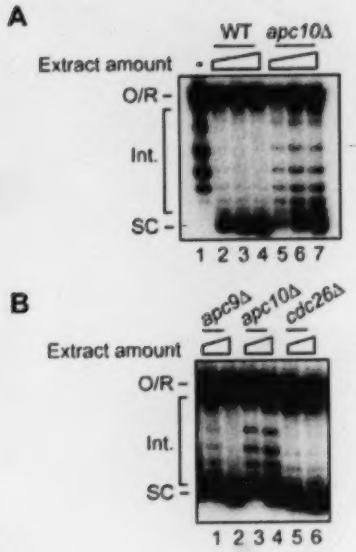


**Figure 5.3. Plasmid supercoiling activity of extract from APC mutants. A.** Wild type strain (WT) compared to *apc10Δ* mutant. 25, 50, and 100 μg of extract protein was assayed for each strain. Lane 1 is the input relaxed plasmid DNA. **B.** Comparison of *apc10Δ* to *apc9Δ* and *cdc26Δ* APC mutants. 25 and 50 μg of extract protein was assayed for each strain. The migration of open circular or relaxed DNA (O/R), intermediate topoisomers (Int.), and highly supercoiled species (SC) is indicated. The experiments presented in this figure were performed by V. Ramaswamy.

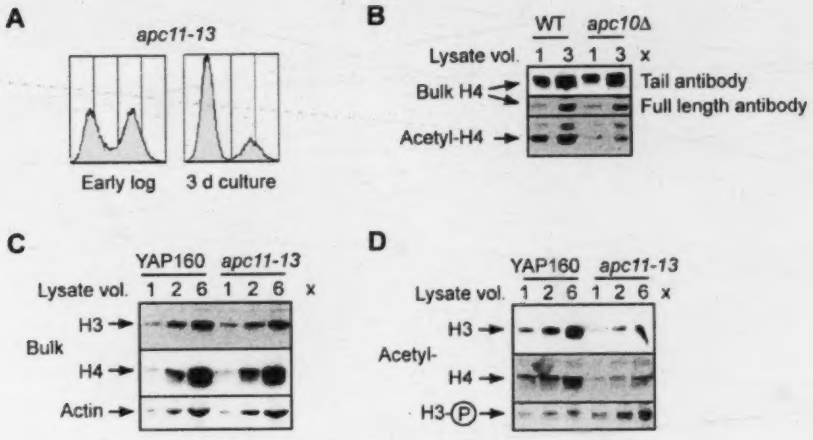


**Figure 5.4. Mutation of a subunit of the catalytic core of the APC disrupts regulation of histone modification state. A.** Flow cytometry profile of *apc11-13* cells during active proliferation (early log phase) and at the point of entry into $G_0$ (3 day culture). **B.** Expression of bulk and tetra-acetylated H4 in 3 day cultures of the indicated strains. Two different antibodies were used to detect bulk H4. **C.** Expression of total H3 and H4 in wild type and *apc11-13* cells cultured for 3 days. Actin was probed as the loading control. **D.** Expression of acetylated H3 and H4 and S10-phosphorylated H3 in wild type and *apc11-13* cells cultured for 3 days. The same samples used in part C were subjected to immunoblot analysis. The experiments presented in this figure were performed by V. Ramaswamy.

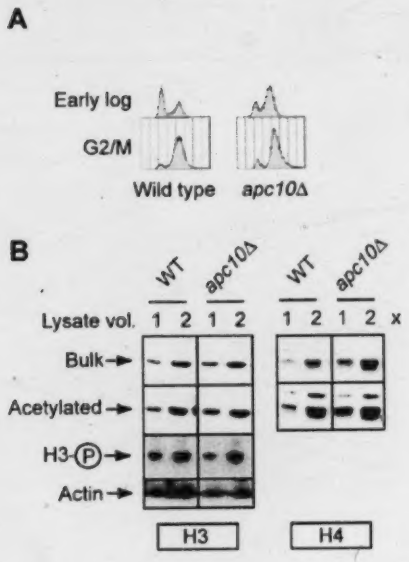


**Figure 5.5. Cell cycle control of histone modification state by the APC. A.** Flow cytometry profiles of wild type and *apc10Δ* cells arrested in $G_2$/M. The profiles of early log phase cultures are shown for comparison. **B.** Levels of acetylated H3 and H4 and S10-phosphorylated H3 in wild type (WT) and *apc10Δ* cells arrested in $G_2$/M. The loading control is actin. The experiments presented in this figure were performed by V. Ramaswamy.

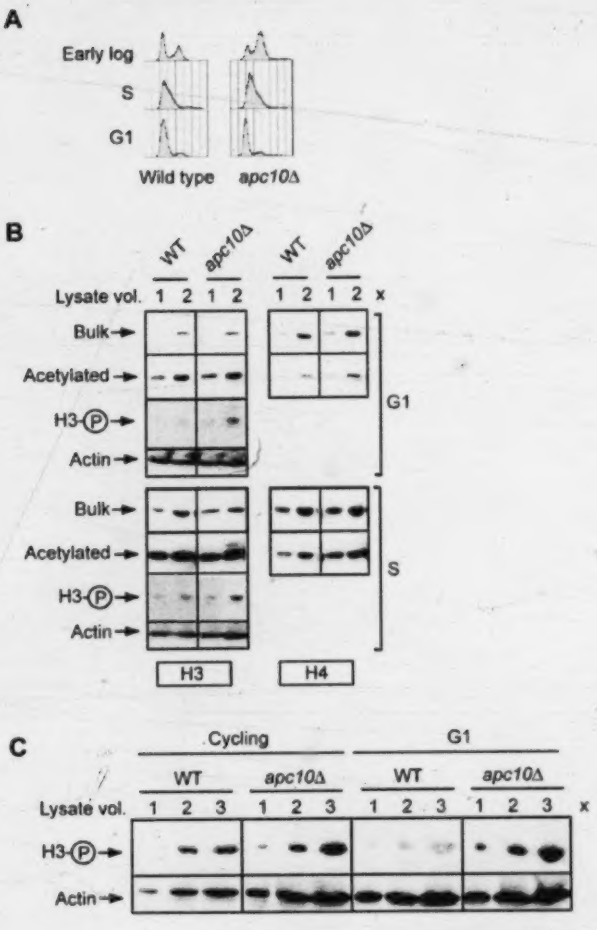


**Figure 5.6. Cell cycle control of histone modification state by the APC. A.** Flow cytometry profiles of wild type and *apc10Δ* cells arrested in $G_1$ or S phase. The profiles of early-log-phase cultures are shown for comparison. **B.** Levels of acetylated H3 and H4 and S10-phosphorylated H3 in wild type and *apc10Δ* cells arrested in $G_1$ or S phase. **C.** Expression of S10-phosphorylated H3 in cycling (early log phase) and $G_1$-arrested cells. Strains are as indicated. The experiments presented in this figure were performed by V. Ramaswamy and J. Williams.

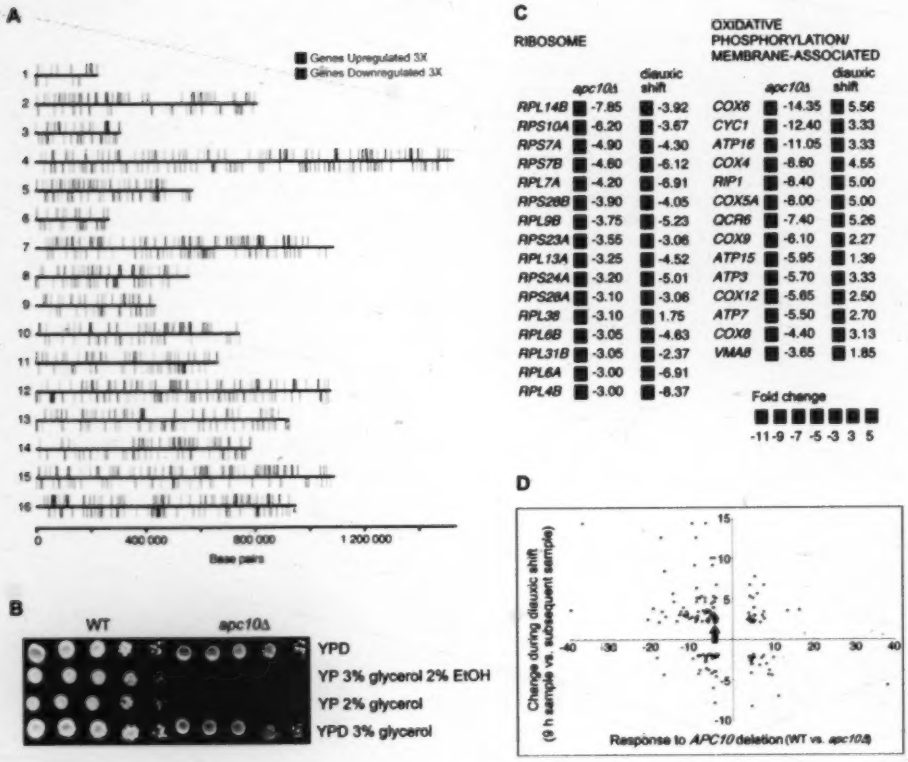


**Figure 5.7. Misregulation of transcription in *apc10Δ* cells. A.** Physical locations of all repressed and induced genes in *apc10Δ* cells. **B.** Serial dilutions of wild type (WT) and *apc10Δ* cells on plates containing different carbon sources. Cells were cultured for 4 days in YPD prior to spotting. **C.** Change in expression levels of genes in two keyword categories in response to deletion of *APC10* in $G_0$ (*apc10Δ*) and to nutrient depletion (diauxic shift) (DeRisi et al., 1997). **D.** Global comparison of the fold change in expression levels of genes that respond both to deletion of *APC10* in $G_0$ (*apc10Δ*) and to nutrient depletion. *GLC7* is indicated by the arrow. The experiment presented in panel B was performed by V. Ramaswamy.

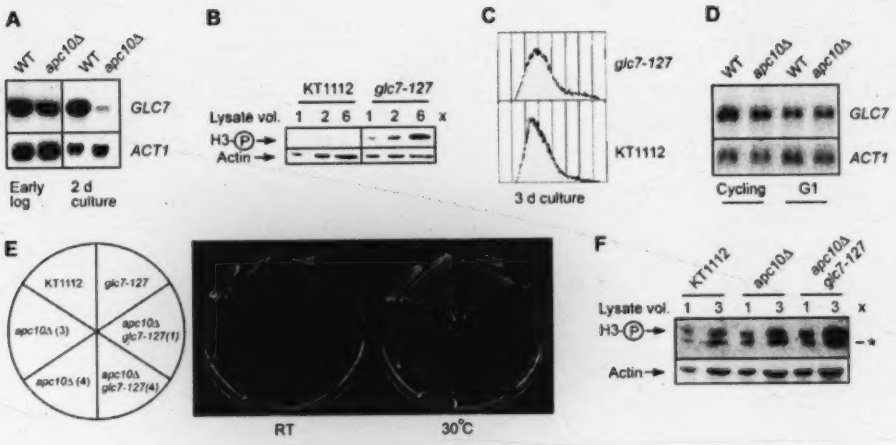


**Figure 5.8. Interactions of the APC with *GLC7*.** **A.** Expression of *GLC7* mRNA in wild type (WT) and *apc10Δ* cells during active proliferation (early log phase) and after 3 days in culture (cells entering $G_0$). 5 μg of total RNA was analyzed; *GLC7* expression levels were normalized to the *ACT1* mRNA control. **B.** S10 phosphorylation state of H3 in *glc7-127* cells entering $G_0$. KT1112 is the wild type strain. **C.** Flow cytometry profile of wild type (KT1112) and *glc7-127* cells after 3 days in culture at the permissive temperature. **D.** Expression of *GLC7* mRNA in cycling (early log phase) and $G_1$-arrested populations of wild type and *apc10Δ* cells. *ACT1* mRNA was probed as the loading control in 5 μg samples of total RNA. **E.** Growth of wild type (KT1112), *glc7-127*, *apc10Δ*, and *apc10Δglc7-127* cells after 3 days on YPD at room temperature (RT) and 30°C. Two different isolates of *apc10Δ* and *apc10Δglc7-127* are shown. **F.** Expression of S10-phosphorylated H3 in populations of wild type (KT1112), *apc10Δ*, and *apc10Δglc7-127* strains after 3 days in culture. The loading control is actin. *, cross-reacting band observed with some lots of the phospho-H3 antibody.

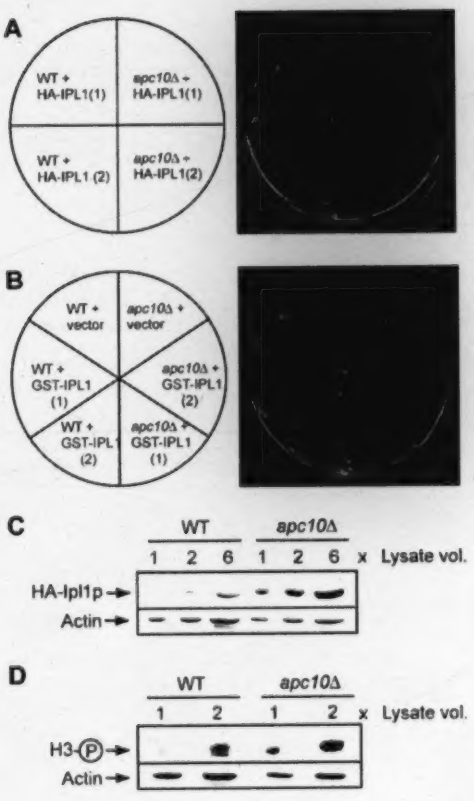


**Figure 5.9. Interactions of the APC with *IPL1*. A.** Growth of wild type (WT) and *apc10Δ* strains carrying the HA-IPL1 plasmid pCC1128. Growth was for 3 days at room temperature on medium lacking leucine. Two different isolates are shown. **B.** Growth of wild type and *apc10Δ* strains carrying the vector (pYEX) or a GST-IPL1 overexpression plasmid. Growth was for 3 days at room temperature on medium lacking uracil. Two different isolates are shown. **C.** Anti-HA immunoblot analysis of $G_1$-arrested wild type and *apc10Δ* strains carrying the HA-IPL1 plasmid pCC1128. The loading control is actin. **D.** Immunoblot analysis of the samples in panel C with antibody against S10-phosphorylated H3.

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# Chapter 6:

# Reflections and Future Directions

**Overall Summary:**

We have characterized components of two distinct mechanisms of global and gene-specific regulation of chromatin metabolism in *S. cerevisiae*. The results of these studies provide further evidence for the central regulatory roles played by chromatin structure and transcription. Because the factors involved are highly conserved, it is likely that the results obtained will apply to chromatin function in higher organisms such as humans.

Our in vivo analysis supports a model in which Asf1 is targeted to the *RNR3* DNA damage response gene in a dynamic manner where it mediates nucleosome-dependent transcriptional regulation. The function of Asf1 at *RNR3* involves histone variant Htz1, transcription initiation factor Bdf1, and chromatin remodeler Isw2.

Biochemical, genetic, and microarray approaches were also employed to characterize a separate mechanism of chromatin alteration, in this case involving physiological regulation of global histone acetylation and phosphorylation state by the APC. We identified the APC as a component of a regulatory network that controls histone modification state and normal programming of transcription in cells executing the $G_0$ program in response to nutrient withdrawal. Our studies link the control of H3 phosphorylation state to APC-dependent regulation of a conserved kinase and phosphatase.

**Determination of the mechanism of Asf1-dependent transcriptional regulation**

Multiple mechanisms may contribute to Asf1-dependent transcriptional regulation. In addition to its possible role in promoter histone deposition/stabilization at *RNR3* (Fig. 3.7, C), Asf1 may organize chromatin structure through establishment of a particular histone acetylation pattern that alters binding of activators or repressors. This may involve the interaction between Asf1 and the SAS H4 acetyltransferase complex (Meijsing and Ehrenhofer-Murray, 2001; Osada et al., 2001), whereby histone deposition by Asf1 is followed by recruitment of this HAT complex, resulting in a specific pattern of H4 acetylation. Alternatively, it may be that association of Asf1 with TFIID at specific genes affects acetylation, as the largest subunit of TFIID, TAF1, is known to possess HAT activity (Mizzen et al., 1996). Furthermore, Asf1 could alter gene-specific

chromatin structure to allow or prevent interactions with components of the transcription initiation and elongation machinery. This may involve the physical association of Asf1 with the SWI/SNF complex (Moshkin et al., 2002).

It should be noted that the mechanisms of recruitment and spreading of Asf1 at *RNR3* are not known. Future work will be aimed at testing if these processes are coupled to the low level of *RNR3* transcription which occurs in normally cycling cells (Cho et al., 1998; Spellman et al., 1998). Although it is not known how Asf1 and Htz1 collaborate in the regulation of *RNR3*, one interesting implication of the observed direct binding of Asf1 to H3/H4 and its association with Bdf1, Htz1 and Swr1, three components of the SWR remodeling complex which can replace Htz1 for nucleosomal H2A (Mizuguchi et al., 2004), is that Asf1 may link the molecular events of H3/H4 tetramer metabolism with steps of chromatin remodeling involving the H2A/H2B dimer. It will be important to identify the entire spectrum of factors involved in recruitment and/or retention of Asf1 to both gene promoters and coding regions. It may be that these targeting proteins also play a regulatory role with respect to transcription and chromatin structure.

The continuous requirement for Asf1-dependent repression may vary at different genes, depending on the nature of the transcriptional regulation. It is possible that the association of Asf1 with target loci is not stable, as is the case for the chromatin remodeler, Isw2. Like Isw2, Asf1 may only be required transiently at its targets (Gelbart et al., 2005). It may be that Asf1 is actually rapidly coming on and off the DNA so that by ChIP it seems to be a permanent resident, when in fact it is highly mobile. Despite possible transient association of Asf1 with its target genes, the chromatin state established may be relatively stable in the absence of Asf1. This is true for Isw2, where the remodeled state of chromatin remains relatively stable upon dissociation of Isw2 (Gelbart et al., 2005).

Our model for repression of *RNR3* by Asf1 makes strong predictions about the regulation of this gene that could be directly tested. For example, we predict that specific targeting of Asf1 to *RNR3* contributes to repression under normal conditions. One way to test this prediction would be to artificially target Asf1 to the *RNR3* promoter and analyze the effect on transcription. This could be accomplished by introducing LexA DNA-binding sites into the *RNR3* promoter and targeting an Asf1-LexA fusion protein to these

transcription factor binding sites. If Asf1 is required for histone stabilization/assembly at *RNR3*, then Asf1 targeted to *RNR3* will repress transcription. It will also be important to determine whether the association between Asf1 and H3/H4 is required for the crosslinking of Asf1 to *RNR3*. Conserved residues in Asf1 involved in binding to H3/H4 have been identified (valine-94, aspartate-54, and asparagine-108; Mousson et al., 2005). Therefore, the requirement for histone binding in Asf1-dependent effects at *RNR3* can be tested by mutating these residues and measuring the ability of the Asf1 mutants to crosslink to *RNR3* by ChIP.

One important issue regarding *RNR3* regulation by Htz1 and Isw2 is that the transcriptional consequence of loss of these factors is rather modest when compared to the level of *RNR3* induction in *asf1Δ* or *crt1Δ* cells (Fig. 4.4, C; Fig. 4.10, A; Huang et al., 1998; Li and Reese, 2001; Zhang and Reese, 2004a, b). Although Isw2 is absolutely required for repositioning of nucleosomes across *RNR3*, disruption of this remodeling does not result in a high level of transcription. J. Reese and colleagues hypothesize that even in the presence of incorrectly spaced nucleosomes at the *RNR3* promoter, Tup1 may still block pre-initiation complex (PIC) formation (Zhang and Reese, 2004a). Therefore, despite the comprehensive model of *RNR3* nucleosome organization and transcriptional regulation that has been developed, there are still many questions left unresolved. Our results add an additional layer of complexity to this regulation, in which a histone chaperone also regulates nucleosome deposition/organization of *RNR3*. In light of the multiple mechanisms involved in nucleosome positioning at *RNR3*, it will be an interesting challenge to understand how the steps which occur at the level of Asf1 are integrated with the other regulatory events that take place at this gene. Clear future challenges will be to further decipher the molecular details of Asf1 regulation at *RNR3* and to assess its possible involvement in physiological regulation of other target genes.

**Specifically targeted Asf1 might play a widespread role in transcriptional regulation**

Asf1 of budding yeast can participate in targeted chromatin remodeling leading to either transcriptional activation (Adkins et al., 2004) or repression (this study), and it is specifically targeted to many genes in addition to *RNR3*. Therefore in yeast Asf1 is likely to be widely used in transcriptional regulation. The same may well be true in higher eukaryotes. Asf1 is conserved in fission yeast and metazoans (Daganzo et al., 2003).

Moshkin et al. (2002) presented evidence that links chromatin remodeling by the Brahma complex of *Drosophila* to nucleosome assembly by Asf1. Like Asf1, Brahma has been implicated in positive and negative regulation of transcription (Martens and Winston, 2003). *Drosophila* Asf1 coimmunoprecipitates with the bromodomain subunit (BRM) of Brahma and is associated with multiple sites along polytene chromosomes (Moshkin et al., 2002; Tyler et al., 2001). Finally, fly *asf1* interacts genetically with the genes encoding BRM and other Brahma subunits (Moshkin et al., 2002). In view of the high conservation of Asf1 and bromodomain proteins (Jeanmougin et al., 1997) and the observed interaction of Asf1 with bromodomain proteins in fly and yeast, the available data hints at a pervasive role for specifically targeted Asf1 in transcriptional regulation in most if not all eukaryotes.

**DNA damage regulation of Asf1 molecules involved in targeted repression of *RNR3***

Our work highlights the complex role of Asf1 in the DNA damage response. Genotoxic stress signals activate global, DNA repair-coupled assembly of nucleosome arrays involving soluble Asf1 and inhibit functions of *RNR3*-localized Asf1 which promote the repressed state of chromatin at *RNR3* (Emili et al., 2001; Mello et al., 2002; Chapter 3). This divergent regulation of Asf1 is likely accommodated by its ability to participate in both replication-coupled and replication-independent chromatin assembly, and its physical interaction with a diverse group of proteins. Being the target of DNA damage signals in both these contexts, Asf1 is in a unique position to coordinate fundamentally dissimilar chromatin remodeling events which are important for cell survival in the face of genotoxic stress. It will be interesting to test in the future if other histone chaperones such as CAF-1 have divergent roles that are regulated by the same physiological signal so as to coordinate steps in chromatin metabolism that have functionally distinct outcomes. It will also be important to determine the function of Asf1 during the cellular response to other DNA damaging agents, such as UV radiation.

**The possibility of a role for Asf1 in DNA repair**

The repair of DNA must occur in the context of chromatin. Deletion of *ASF1* or CAF-1 subunits leads to sensitivity to DNA damaging agents, due to a requirement for these histone chaperones in repair-coupled assembly (Tyler et al., 1999; Game and Kaufman, 1999; Kaufman et al., 1997; Gaillard et al., 1996; Moggs et al., 2000). Histone

modifications play a critical role in DNA damage-signaling pathways and DNA repair (Downs et al., 2000; Fernandez-Capetillo et al., 2004; Nakamura et al., 2004; Bird et al., 2002; Jazayeri et al., 2004). In addition to the role played by chromatin modifying complexes, chromatin remodeling factors have also recently been shown to play a central role in DNA repair through reorganization of chromatin structure in order to facilitate access of the repair machinery (Downs et al., 2004; van Attikum et al., 2004; Morrison et al., 2004; Peterson and Cote, 2004; Bilsland and Downs, 2005). The NuA4 HAT complex, and SWR1 and INO80 chromatin remodeling complexes are recruited to sites of DNA DSBs by phosphorylated H2A (H2A.X). Following chromatin reconfiguration and repair, chromatin assembly is required to restore the original chromatin structure. The interactions observed between Asf1 and SWR1 components, Swr1, Htz1, and Bdf1 (Fig. 4.6, B), may target Asf1 to sites of DNA damage where it promotes efficient chromatin assembly following DNA repair. Because all of the events involved in DNA repair, from sensing of damage to processing of the break, occur in the context of chromatin, it will be critical to understand the role that histone chaperones such as Asf1 play in these events.

**Determination of the overall functional significance of histone phosphorylation**

The significance of global (non-targeted) histone modification remains elusive. A considerable body of evidence suggests that global H3 phosphorylation in *Tetrahymena* and metazoans is important for chromosome condensation. The fact that similar kinases and phosphatases control global H3 phosphorylation state in yeast and vertebrates heightens the expectation that H3 phosphorylation also regulates chromosome condensation in yeast. Mutation of H3-S10 to alanine however has no effect on chromosome dynamics in cycling yeast cells, even when another nearby site of phosphorylation in vivo (S28) is also changed to alanine (Hsu et al., 2000). Importantly, this result does not exclude the possibility that global H3 phosphorylation regulates chromosome structure in $G_0$. For example, the level of H3 phosphorylation set by APC-dependent events in $G_1$ may be just below a threshold required to change the global structure of chromosomes. Persistent APC-dependent H3 dephosphorylation in the context of global histone deacetylation might then trigger global changes in chromosome structure when the $G_0$ program is initiated. Accordingly studies of chromosome

metabolism in yeast that focus on histone modification during the $G_1$ to $G_0$ transition may shed new light on the control of chromosome structure in higher organisms.

It is possible that APC-dependent changes in the global level of H3-S10 phosphorylation influence global acetylation levels. For example, there is a mechanistic link between global levels of H3-S10 phosphorylation and H3-K9 acetylation (Lo et al., 2000, 2001; Edmondson et al., 2002). Increased levels of H3-S10 phosphorylation correlate with decreased acetylation of K9 on H3 (Edmondson et al., 2002), and it will be important to test if APC-dependent regulation of global histone acetylation levels is influenced by the global phosphorylation level of H3-S10. Global levels of histone phosphorylation and acetylation represent the sum of local (gene-specific) and non-targeted events (Fischle et al., 2003). Therefore, the APC may also be involved in regulation of histone phosphorylation and acetylation state at specific target loci, including those genes that require Apc10 for proper activation or repression during stationary phase identified in our microarray experiment. ChIP analysis using antibodies specific for unmodified and modified histones in wild type and *apc10Δ* cells would address this possibility.

**Implications for disease treatment**

Carcinogenesis often involves genome instability which ultimately results in the gain or loss of chromosomes. There is evidence in the literature that Asf1 is required for proper maintenance of genome stability in *S. cerevisiae* (Myung et al., 2003; Prado et al., 2004). Interestingly, the genes encoding the human Asf1 orthologs, Asf1a and Asf1b, are located in regions reported to be deleted in some breast cancer cell lines (6q22 and 19p13 respectively; Oesterreich et al., 2001; Sheng et al., 1996). It is therefore possible that the proteins encoded by these genes are involved in the suppression of tumorigenesis.

Mutations in APC subunits have been identified in human colon cancer cells and overexpression of several APC substrates has been demonstrated in human cancers (Wang et al., 2003; Wasch and Engelbert, 2005). These substrates include pituitary tumor-transforming gene (PTTG; securin in budding yeast), Aurora A, and cyclin B1 (Clb2 in budding yeast; Wang et al., 1997; Bischoff et al., 1998; Heaney et al., 2000; Soria et al., 2000; Sarafan-Vasseur et al., 2002). These results suggest the possibility that the APC and/or these substrates would be suitable candidate targets for cancer

prevention, prognosis, and therapy. For example, small molecule therapeutics that destabilize these APC substrates may be effectively used as anticancer agents. However, one considerable challenge is to find therapeutic agents that are highly specific for tumor cells in order to minimize toxicity in the cancer patient.

The balance of H3 phosphorylation and dephosphorylation is vitally important for chromosome segregation during mitosis and meiosis. Loss of regulation of human Aurora kinases has been implicated in tumorigenesis, with them being overexpressed in a variety of cancers which include colorectal and breast cancer cell lines (Nigg, 2001; Sen et al., 1997; Gopalan et al., 1997; Bischoff et al., 1998; Tatsuka et al., 1998, Zhou et al., 1998). For example, elevated Aurora A levels have been detected in polyploid cancer cells undergoing an aberrant mitosis without cytokinesis (Meraldi et al, 2002). Ectopic expression of Aurora A causes centrosome amplification and aneuploidy in cultured cells (Zhou et al., 1998). Therefore, regulation of Aurora levels appears to be critical to the maintenance of genome stability and this may involve APC-dependent regulation of Aurora levels. Furthermore, genes encoding human Aurora kinases map to chromosomal regions that are frequently altered in tumors (Aurora A, 20q13; Aurora B, 17p13; Aurora C, 19q13). Taken together, these results suggest that regulation of mitosis by Aurora signaling may be a target for cancer treatment. In mammalian cells, Aurora B overexpression results in hyperphosphorylation of histone H3 on serine 10 and missegregation of chromosomes (Ota et al., 2002). It remains to be determined whether oncogenesis correlates with hyperphosphorylation of H3-S10.

Changes in chromatin architecture due to mutations in human proteins involved in regulation of histone acetylation are also associated with disease. Alterations in both HATs and HDACs have been identified in tumor cells and may contribute to the altered gene expression found in many cancers. Mutations in the methyl-CpG binding proein 2 (MECP2) gene cause a neurodevelopmental disorder called Rett syndrome (RTT; Amir et al., 1999). MeCP2 interacts with HDAC-containing complexes to act as a global transcriptional inhibitor (Jones et al., 1998; Nan et al., 1998), and MeCP2 mutant mice display elevated levels of H3 acetylation (Shahbazian et al., 2002). The development of specific tumors also correlates with the failure of transcriptional repression due to disregulation of HDAC expression in humans (Cress and Seto, 2000). In contrast, the

expression of acetylated H4 is reduced in many gastric and colorectal cancers (Yasui et al., 2003). Taken together, these results suggest that histone acetylation may be a promising target for therapy, and perhaps even prevention in some cases. Consequently, it is of vital importance to determine the mechanisms by which HAT and HDAC function is regulated as these enzymes represent targets for cancer therapeutic agents. Our results suggest that the APC may be involved in HAT and/or HDAC regulation, and therefore targeting the APC may also be an effective means of treating disorders that arise due to misregulation of histone acetylation.

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**Appendix I:**

**Microarray Data for CAF deletion mutants**

**Appendix Table 7.1.** Genes most significantly upregulated in *asf1*Δ cells. The average fold change result from two independent experiments is provided.

| Gene | Avg. Fold Δ | ORF and Description |
|---|---|---|
| *HSP12* | 69 | YFL014W 12 kDa heat shock protein |
| | 38 | YGR052W similarity to ser/thr protein kinases |
| *CRC1* | 34 | YOR100C similarity to mitochondrial carrier proteins |
| *BNA2* | 32 | YJR078W sim. to mammalian indoleamine dioxygenase |
| | 32 | YNL194C strong similarity to YDL222c |
| | 31 | YJR079W questionable ORF |
| *SIP18* | 29 | YMR175W protein of unknown function |
| | 23 | YJL045W sim. to succinate dehydrogenase flavoprotein |
| | 20 | YPL230W Up in StarVation |
| | 18 | YPLWDELTA11 Ty1 LTR |
| *DDR2* | 17.7 | YOL053C-A DNA Damage Responsive |
| | 17.7 | YPL136W questionable ORF |
| *DAL1* | 16.6 | YIR027C allantoinase |
| *INH1* | 13 | YDL181W ATPase inhibitor |
| | 13 | YMR107W hypothetical protein |
| *EMI5* | 12.6 | YOL071W similarity to hypothetical *S. pombe* protein |
| *GPH1* | 12.6 | YPR160W Glycogen phosphorylase |
| | 11.3 | YOL163W similarity to P. putida phthalate transporter |
| | 10.2 | YKL162C-A hypothetical protein identified by SAGE |
| | 9.8 | YDR070C hypothetical protein |
| | 9.8 | YLR312C hypothetical protein |
| *CYC7* | 9.5 | YEL039C iso-2-cytochrome c |
| *HUG1* | 9.5 | YML058w-a identified by SAGE |
| *SLF1* | 9.2 | YDR515W reg. Cu-dep. mineralization of copper sulfide |
| *PIR3* | 9.2 | YKL163W Protein containing tandem internal repeats |
| *RIM4* | 8.9 | YHL024W RNA binding domain with |
| *GPG1* | 8.9 | YGL121C hypothetical protein |
| | 8.9 | YJL037W similarity to hypothetical protein YJL038c |
| *PES4* | 8.9 | YFR023W poly(A) binding protein |
| *MIP6* | 8 | YHR015W PolyA-binding protein |
| *GSP2* | 7.7 | YOR185C GTP binding protein |
| *SGA1* | 7.7 | YIL099W intracellular glucoamylase |
| | 7.5 | YCL042W questionable ORF |
| | 7 | YMR118C strong similarity to succinate dehydrogenase |
| *HXT4* | 6.7 | YHR092C High-affinity glucose transporter |
| *HSP26* | 6.7 | YBR072W heat shock protein 26 |
| *INO1* | 6.5 | YJL153C L-myo-inositol-1-phosphate synthase |
| *RNR3* | 6.5 | YIL066C Ribonucleotide reductase |
| | 6.3 | TM(CAU)J3 tRNA-Met |
| *STL1* | 6.3 | YDR536W sugar transporter-like protein |

| | | |
|---|---|---|
| | 6.3 | YDR034W-B identified by SAGE expression analysis |
| *YGP1* | 6.3 | YNL160W YGP1 encodes gp37, a glycoprotein |
| *MCH2* | 6.1 | YKL221W sim. to human X-linked PEST transporter |
| | 6.1 | YLR327C strong similarity to Stf2p |
| | 6.1 | YGR043C strong similarity to transaldolase |
| | 5.9 | YJL161W hypothetical protein |
| *ARG1* | 5.9 | YOL058W arginosuccinate synthetase |
| *NCA3* | 5.9 | YJL116C regulates proper expression of subunits 6 and 8 |
| *SNO4* | 5.9 | YMR322C strong similarity to YPL280w and YOR391c |
| *JEN1* | 5.9 | YKL217W carboxylic acid transporter protein homolog |
| | 5.9 | YOL037C questionable ORF |
| *PCL5* | 5.7 | YHR071W G1/S cyclin (weak) |
| *FBP1* | 5.7 | YLR377C fructose-1,6-bisphosphatase |
| *TFS1* | 5.7 | YLR178C suppressor of cdc25 |
| *HOR2* | 5.7 | YER062C DL-glycerol-3-phosphatase |
| *PRY1* | 5.7 | YJL079C Similar to plant PR-1 class of pathogen proteins |
| | 5.7 | YFL030W similarity to several transaminases |
| | 5.7 | YBL065W questionable ORF |
| | 5.5 | YNR064C similarity to 1-chloroalkane halidohydrolase |
| | 5.3 | YGR243W strong similarity to hypothetical protein |
| *YRO2* | 5.3 | YBR054W Homolog to HSP30 heat shock protein YRO1 |
| *DIT1* | 5.3 | YDR403W first enzyme in dityrosine synthesis |
| | 5.3 | YKL086W hypothetical protein |
| *YPS5* | 5.3 | YGL259W GPI-anchored aspartic protease |
| | 5.3 | YOL155C similarity to glucan 1,4-alpha-glucosidase |
| | 5.3 | YPR077C questionable ORF |
| | 5.3 | YIR043C putative pseudogene |
| *NPR2* | 5.1 | YEL062W PEST sequence-containing protein |
| *SNR54* | 5.1 | SNR54 snRNA |
| *PGM2* | 5.1 | YMR105C Phosphoglucomutase |
| *RTN2* | 5.1 | YDL204W similarity to hypothetical protein YDR233c |
| *MSC1* | 5.1 | YML128C C-terminal part causes growth inhibition |
| | 5.1 | YHR087W hypothetical protein |
| *SHC1* | 5.1 | YER096W sporulation-specific homolog of csd4 |
| *HXK1* | 5.1 | YFR053C Hexokinase I (PI) (also called Hexokinase A) |
| | 4.9 | YLR053C hypothetical protein |
| | 4.9 | YDR018C similarity to hypothetical protein YBR042c |
| *COX9* | 4.9 | YDL067C Subunit VIIa of cytochrome c oxidase |
| *IDP1* | 4.9 | YDL066W NADP-specific isocitrate dehydrogenase |
| *SPI1* | 4.9 | YER150W similarity to cell surface glycoprotein Sed1p |
| | 4.9 | YOL155C similarity to glucan 1,4-alpha-glucosidase |
| *HXT9* | 4.9 | YJL219W High-affinity hexose transporter |
| *SPS19* | 4.9 | YNL202W peroxisomal 2,4-dienoyl-CoA reductase |
| | 4.9 | YNR034w-a hypothetical protein |
| *SOL4* | 4.9 | YGR248W similar to SOL3 |
| *GAP1* | 4.8 | YKR039W general amino acid permease |

| | | |
|---|---|---|
| *CYC1* | 4.8 | YJR048W iso-1-cytochrome c |
| *STF1* | 4.8 | YDL130W-A ATPase stabilizing factor |
| *YPS6* | 4.8 | YIR039C GPI-anchored aspartic protease |
| | 4.6 | YDR374C similarity to hypothetical *A. thaliana* protein |
| *GAD1* | 4.4 | YMR250W similarity to glutamate decarboxylases |
| *UBI4* | 4.4 | YLL039C ubiquitin |
| *COX4* | 4.4 | YGL187C subunit IV of cytochrome c oxidase |
| *TKL2* | 4.4 | YBR117C transketolase, homologous to tkl1 |
| | 4.4 | YBL048W hypothetical protein |
| *NDJ1* | 4.3 | YOL104C Involved in meiotic chromosome segregation |
| *LOT6* | 4.3 | YLR011W similarity to *E. coli* 20.4 kDa protein |
| *BOP2* | 4.3 | YLR267W Bypass of PAM1 |
| *MED1* | 4.3 | YPR070W hypothetical protein |
| | 4.3 | YHR209W similarity to hypothetical protein YER175c |
| *PET10* | 4.3 | YKR046C hypothetical protein |
| *ARG3* | 4.3 | YJL088W Ornithine carbamoyltransferase |
| *CTT1* | 4.3 | YGR088W cytoplasmic catalase T |
| *MOH1* | 4.3 | YBL049W hypothetical protein |
| | 4.1 | YBR109w-a questionable ORF - upstream ORF of ALG1 |
| *RNY1* | 4.1 | YPL123C similarity to ribonucleases |
| *XBP1* | 4.1 | YIL101C DNA-binding transcriptional repressor |
| *NAT5* | 4.1 | YOR253W hypothetical protein |
| | 4.1 | YLR149C hypothetical protein |
| | 4.1 | YDR042C hypothetical protein |
| | 4.1 | YBR147W similarity to hypothetical protein YOL092w |
| *SWI4* | 4 | YER111C transcription factor |
| *RGT1* | 4 | YKL038W transcriptional repressor and activator |
| | 4 | YLR004C similarity to allantoate transport protein |
| | 4 | YBR116C questionable ORF |
| *GLC3* | 4 | YEL011W 1,4-glucan-6-(1,4-glucano)-transferase |
| *DMC1* | 4 | YER179W meiosis-specific protein |
| *ARG8* | 3.9 | YOL140W Acetylornithine aminotransferase |
| *MLS1* | 3.9 | YNL117W carbon-catabolite sensitive malate synthase |
| *SOM1* | 3.9 | YEL059C-A high copy suppressor of imp1 mutation |
| *PUT4* | 3.9 | YOR348C putative proline-specific permease |
| | 3.9 | YOL162W similarity to hypothetical protein YIL166c |
| *ATP2* | 3.9 | YJR121W F(1)F(0)-ATPase complex beta subunit |
| *HBT1* | 3.9 | YDL223C weak similarity to mucin |
| *ALD3* | 3.9 | YMR169C Aldehyde Dehydrogenase (NAD(P)+) |
| | 3.9 | YIR016W weak similarity to YOL036w |
| | 3.7 | TQ(UUG)E2 tRNA-Gln |
| *TIS11* | 3.7 | YLR136C zinc finger containing homolog of TIS11 gene |
| *PRB1* | 3.7 | YEL060C vacuolar protease B |
| | 3.7 | YOL047C similarity to hypothetical protein YAL018c |
| *UGA2* | 3.7 | YBR006W Probable aldehyde dehydrogenase |
| *FIG1* | 3.7 | YBR040W integral membrane protein |

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|---|---|---|
| | 3.7 | YBL064C Homolog to thiol-specific antioxidant |
| *QCR2* | 3.7 | YPR191W ubiquinol cytochrome-c reductase protein 2 |
| *MEP2* | 3.7 | YNL142W Ammonia transport protein |
| *YTP1* | 3.7 | YNL237W Yeast putative Transmembrane Protein |
| | 3.7 | YCL049C hypothetical protein |
| *CYB2* | 3.7 | YML054C Cytochrome b2 |
| | 3.7 | YGR153W hypothetical protein |
| *SBE2* | 3.6 | YDR351W required for bud growth |
| | 3.6 | TR(UCU)E tRNA-Arg |
| | 3.6 | SNR14 snRNA R14 |
| | 3.6 | YBR203W hypothetical protein |
| | 3.6 | YPL017C sim. to dihydrolipoamide dehydrogenases |
| | 3.6 | TH(GUG)M tRNA-His |
| *MBR1* | 3.6 | YKL093W MBR1 protein precursor |
| *RDN5-1* | 3.6 | RDN5-1 5S ribosomal RNA |
| *UBC5* | 3.6 | YDR059C ubiquitin-conjugating enzyme |
| *AUT4* | 3.6 | YCL038C Membrane transporter |
| *PAI3* | 3.6 | YMR174C Cytoplasmic inhibitor of proteinase Pep4p |
| *GRE1* | 3.6 | YPL223C Induced by osmotic stress |
| *MSN4* | 3.6 | YKL062W zinc finger protein |
| | 3.6 | YNR062C similarity to H.influenzae L-lactate permease |
| *ADH2* | 3.6 | YMR303C alcohol dehydrogenase II |
| | 3.5 | TR(UCU)D tRNA-Arg |
| *MET28* | 3.5 | YIR017C Transcriptional activator of sulfur aa metab. |
| | 3.5 | SNR72 snRNA |
| | 3.5 | YLL020C questionable ORF |
| *ECM4* | 3.5 | YKR076W ExtraCellular Mutant |
| *GDB1* | 3.5 | YPR184W sim. to human 4-alpha-glucanotransferase |
| | 3.5 | YAL061W similarity to alcohol/sorbitol dehydrogenase |
| *HXT5* | 3.5 | YHR096C hexose transporter |
| | 3.5 | YBR012C hypothetical protein |
| *CPS1* | 3.5 | YJL172W carboxypeptidase yscS |
| *HCM1* | 3.5 | YCR065W Transcription factor (fork head domain) |
| *MAL11* | 3.5 | YGR289C alpha-glucoside transporter |
| *FUN34* | 3.5 | YNR002C Putative transmembrane protein |
| | 3.4 | TH(GUG)E1 tRNA-His |
| | 3.4 | YLL020C questionable ORF |
| | 3.4 | YNR073C sim. to *E. coli* D-mannonate oxidoreductase |
| *FCY22* | 3.4 | YER060w-A purine-cytosine permease |
| *UIP4* | 3.4 | YPL186C weak similarity to *Xenopus* protein xlgv7 |
| | 3.4 | YNL195C hypothetical protein |
| | 3.4 | YGL188C hypothetical protein |
| | 3.4 | YER121W hypothetical protein |
| *CIT3* | 3.4 | YPR001W Mitochondrial isoform of citrate synthase |
| | 3.4 | YGL010W similarity to hypothetical *S. pombe* protein |
| | 3.4 | YFL054C similarity to channel proteins |

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|---|---|---|
| | 3.2 | YMRCDELTA14 Ty1 LTR |
| *BNS1* | 3.2 | YGR230W questionable ORF |
| | 3.2 | YIR042C weak similarity to *B. licheniformi* protein P20 |
| | 3.2 | YFL057C similarity to aryl-alcohol dehydrogenases |
| | 3.2 | YNL200C strong similarity to human TGR-CL10C |
| *RNR2* | 3.2 | YJL026W Ribonucleotide reductase |
| *SKN1* | 3.2 | YGR143W encodes a predicted type II membrane protein |
| | 3.2 | TQ(UUG)E1 tRNA-Gln |
| | 3.2 | YPL067C hypothetical protein |
| | 3.2 | TQ(UUG)C tRNA-Gln |
| *PBI2* | 3.2 | YNL015W Proteinase inhibitor I2B (PBI2) |
| *GSY2* | 3.2 | YLR258W Glycogen synthase |
| | 3.2 | YPL264C strong similarity to YMR253c |
| | 3.2 | YHL006w-a questionable ORF |
| *DCS2* | 3.2 | YOR173W strong similarity to YLR270w |
| *HAC1* | 3.1 | YFL031W bZIP (basic-leucine zipper) protein |
| | 3.1 | TQ(UUG)D1 tRNA-Gln |
| *QCR10* | 3.1 | YHR001W-A subunit of a oxidoreductase complex |
| | 3.1 | YOR186W hypothetical protein |
| | 3.1 | M23316 SGD:YEL024C Yeast *S. cerevisiae* RIP1. |
| *RIP1* | 3.1 | YEL024W Rieske iron-sulfur protein - mitochondrial |
| *ADR1* | 3.1 | YDR216W positive transcriptional regulator of ADH2 |
| *VID24* | 3.1 | YBR105C fructose-1,6-bisphosphatase degradation |
| | 3.1 | YNR014W similarity to hypothetical protein YMR206w |
| | 3.1 | YHR054C weak similarity to YOR262w |
| *THI4* | 3.1 | YGR144W component of a biosynthetic pathway |
| *VPS73* | 3.1 | YGL104C similarity to glucose transport proteins |
| *SRT1* | 3.1 | YMR101C similarity to YBR002c |
| | 3.1 | YJL163C hypothetical protein |
| *ODC1* | 3.1 | YPL134C similarity to ADP/ATP carrier proteins |
| *GPM2* | 3.1 | YDL021W Similar to GPM1 (phosphoglycerate mutase) |
| | 3.1 | YJR020W questionable ORF |
| *MRP4* | 3.1 | YHL004W mitochondrial ribosomal protein |
| | 3 | YLR077W weak similarity to *Xenopus* RCC1 protein |
| | 3 | YLR297W weak similarity to *V. vulnificus* VvpC protein |
| *COX5A* | 3 | YNL052W Cytochrome-c oxidase chain Va |
| | 3 | YJR115W similarity to hypothetical protein YBL043w |
| | 3 | YJL225C sim. to members of the Sir1p/Tip1p family |
| | 3 | TR(UCU)K tRNA-Arg |
| *CIN5* | 3 | YOR028C bZIP protein, can activate transcription |
| *RDN37-1* | 3 | RDN37-1 35S ribosomal RNA |
| *HAC1* | 3 | YFL031W bZIP (basic-leucine zipper) protein |
| *RPM2* | 3 | YML091C subunit of mitochondrial RNase P |
| *MCR1* | 3 | YKL150W NADH-cytochrome b5 reductase |
| | 3 | YKL161C probable serine/threonine protein kinase |
| *LSC2* | 3 | YGR244C Succinate-CoA Ligase (ADP-Forming) |

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|---|---|---|
| *OPY2* | 3 | YPR075C imparts Far- phenotype |
| *PHM7* | 3 | YOL084W similarity to *A. thaliana* hyp1 protein |
| *URA10* | 3 | YMR271C Orotate phosphoribosyltransferase 2 |
| *BNA1* | 3 | YJR025C 3-hydroxyanthranilic acid dioxygenase |
| *HXT6* | 3 | YDR343C Hexose transporter |
| *LAP4* | 3 | YKL103C vacuolar aminopeptidase ysc1 |
| *DAL4* | 3 | YIR028W allantoin permease |
| *PHM8* | 3 | YER037W similarity to hypothetical protein YGL224c |
| *DTR1* | 3 | YBR180W Probable resistance protein |
| | 3 | YMR209C hypothetical protein |

**Appendix Table 7.2.** Genes upregulated in *cac1*Δ cells. The average fold change result from two independent experiments is provided.

| Gene | Avg. Fold Δ | ORF and Description |
|---|---|---|
| | 55.7 | YGR111W weak similarity to mosquito carboxylesterase |
| | 39.4 | YJR079W questionable ORF |
| | 26.9 | YJR078W sim. to mammalian indoleamine 2,3-dioxygenase |
| *FIG1* | 26 | YBR040W integral membrane protein |
| | 22.6 | YOL162W strong similarity to hypothetical protein YIL166c |
| | 21.9 | YNR064C sim. to *R. capsulatus* 1-chloroalkane halidohydrolase |
| *SPO1* | 21.9 | YNL012W strong similarity to phospholipase B |
| | 19 | YNL335W similarity to *M. verrucaria* cyanamide hydratase |
| | 14.9 | YPR077C questionable ORF |
| | 14.9 | YBR178W questionable ORF |
| | 14.9 | YOL163W similarity to P. *putida* phthalate transporter |
| *THI11* | 13.9 | YJR156C Thiamine biosynthetic enzyme |
| | 12.6 | YMR322C strong similarity to YPL280w and YOR391c |
| | 11.3 | YMR107W hypothetical protein |
| *FYV12* | 11.3 | YOR183W hypothetical protein |
| | 11.3 | YBL065W questionable ORF |
| *ADH2* | 10.9 | YMR303C alcohol dehydrogenase II |
| | 10.9 | YOL047C weak similarity to hypothetical protein YAL018c |
| *CRC1* | 10.6 | YOR100C similarity to mitochondrial carrier proteins |
| | 10.6 | YGR066C similarity to hypothetical protein YBR105c |
| | 10.2 | YMR118C strong similarity to succinate dehydrogenase |
| *FDH1* | 9.8 | YOR388C Protein with similarity to formate dehydrogenases |
| *DAL1* | 9.8 | YIR027C allantoinase |
| *PRM2* | 8.9 | YIL037C hypothetical protein |
| | 8.3 | YLL057C similarity to *E. coli* dioxygenase |
| | 8.3 | YOR387C strong similarity to YGL258w |
| | 7.7 | YGL258W strong similarity to hypothetical protein YOR387c |
| | 7.7 | YJL045W similarity to succinate dehydrogenase flavoprotein |
| *SPR3* | 7.7 | YGR059W a sporulation-specific homologue of yeast CDC3 |
| *HUG1* | 7.5 | YML058w-a identified by SAGE |
| *DTR1* | 7.2 | YBR180W Probable resistance protein |
| *JEN1* | 7 | YKL217W carboxylic acid transporter protein homolog |
| | 6.5 | YLR149C hypothetical protein |
| *TKL2* | 6.5 | YBR117C transketolase, homologous to tkl1 |
| | 6.3 | YNL034W nearly identical to YNL018c |
| *HXT9* | 6.3 | YJL219W High-affinity hexose transporter |
| *STE3* | 6.1 | YKL178C a factor recptor |
| *PRM1* | 5.9 | YNL279W hypothetical protein |
| *HSP12* | 5.9 | YFL014W 12 kDa heat shock protein |
| | 5.7 | YDR374C similarity to *A. thaliana* protein BAC F21M12 |

| | | |
|---|---|---|
| | 5.5 | YJL037W strong similarity to hypothetical protein YJL038c |
| *SPS19* | 5.5 | YNL202W peroxisomal 2,4-dienoyl-CoA reductase |
| | 5.5 | YMR244W similarity to Uth1p, Nca3p, YIL123w and Sun4p |
| | 5.5 | YPR177C questionable ORF |
| | 5.3 | YGL258W strong similarity to hypothetical protein YOR387c |
| | 5.3 | YIR043C putative pseudogene |
| | 5.3 | YJR119C similarity to human retinoblastoma binding protein 2 |
| | 5.3 | YGL262W similarity to hypothetical protein YER187w |
| | 4.9 | YBR116C questionable ORF |
| *MCH2* | 4.9 | YKL221W similarity to human X-linked PEST transporter |
| | 4.9 | YJR079W questionable ORF |
| | 4.8 | YDR034W-B identified by SAGE expression analysis |
| | 4.8 | YBL070C questionable ORF |
| *PTR2* | 4.6 | YKR093W Peptide transporter |
| *THI2* | 4.6 | YBR240C Probable Zn-finger protein |
| | 4.3 | YNL226W questionable ORF |
| | 4.3 | YHL006w-a questionable ORF |
| | 4.1 | YAL018C 3 transmembrane domains |
| *GND2* | 4.1 | YGR256W 6-phosphogluconate dehydrogenase |
| | 4.1 | YPL136W questionable ORF |
| | 4.1 | YDL218W weak similarity to hypothetical protein YNR061c |
| *HEF3* | 4.1 | YNL014W translation elongation factor eEF3 homolog |
| | 4.1 | YLR031W similarity to hypothetical protein YMR124w |
| *PGU1* | 4 | YJR153W Endo-polygalacturonase |
| *GPH1* | 4 | YPR160W Glycogen phosphorylase |
| | 4 | YBR116C questionable ORF |
| *INO1* | 4 | YJL153C L-myo-inositol-1-phosphate synthase |
| *GAL7* | 3.9 | YBR018C galactose-1-phosphate uridyl transferase |
| | 3.9 | YHR015W PolyA-binding protein |
| *DIT1* | 3.9 | YDR403W first enzyme in dityrosine synthesis |
| *YPS5* | 3.9 | YGL259W GPI-anchored aspartic protease |
| | 3.9 | YHR126C hypothetical protein |
| | 3.7 | YNR062C similarity to *H. influenzae* L-lactate permease (lctP) |
| *FKS3* | 3.7 | YMR306W Protein with similarity to Gls1p and Gls2p |
| *FBP1* | 3.7 | YLR377C fructose-1,6-bisphosphatase |
| | 3.6 | YPR064W hypothetical protein |
| | 3.6 | YIL177C strong similarity to subtelomeric encoded proteins |
| *GAL10* | 3.6 | YBR019C UDP-glucose 4-epimerase |
| *SMA1* | 3.6 | YPL027W hypothetical protein |
| | 3.5 | YFR032C similarity to *S. pombe* polyadenylate-binding protein YPR112c |
| | 3.5 | YDL222C strong similarity to hypothetical protein YNL194c |
| | 3.5 | YNR073C similarity to *E. coli* D-mannonate oxidoreductase |
| *OPT2* | 3.5 | YPR194C similarity to *S. pombe* isp4 protein |
| *MLS1* | 3.5 | YNL117W carbon-catabolite sensitive malate synthase |
| | 3.5 | YCR045C Protease |
| *GRE1* | 3.5 | YPL223C Induced by osmotic stress |

| | | |
|---|---|---|
| *CYB2* | 3.5 | YML054C Cytochrome b2 |
| | 3.4 | YAR053W predicted membrane protein |
| *HSP26* | 3.4 | YBR072W heat shock protein 26 |
| *SSE2* | 3.4 | YBR169C HSP70 family member, highly homologous to Sse1p |
| *ADY2* | 3.4 | YCR010C similarity to *Y. lipolytica* GPR1 protein and Fun34p |
| | 3.4 | YGL081W hypothetical protein |
| *SEO1* | 3.2 | YAL067C Suppressor of Sulfoxyde Ethionine resistance |
| *RNR3* | 3.2 | YIL066C Ribonucleotide reductase |
| *AGA1* | 3.2 | YNR044W anchorage subunit of a-agglutinin |
| *SPS4* | 3.2 | YOR313C sporulation-specific protein |
| *YPS6* | 3.2 | YIR039C GPI-anchored aspartic protease |
| | 3.2 | YPL113C similarity to glycerate dehydrogenases |
| *ECM8* | 3.2 | YBR076W ExtraCellular Mutant |
| | 3.1 | YMR007W hypothetical protein |
| *OYE3* | 3.1 | YPL171C NAD(P)H dehydrogenase |
| | 3.1 | YDL026W questionable ORF |
| *PDC5* | 3.1 | YLR134W pyruvate decarboxylase |
| | 3.1 | YDR366C similarity to YOL106w and YER181c |
| *BIO3* | 3 | YNR058W 7,8-diamino-pelargonic acid aminotransferase |
| | 3 | YGL121C hypothetical protein |
| *SPS100* | 3 | YHR139C sporulation-specific wall maturation protein |
| *DAN1* | 3 | YJR150C Protein induced during anaerobic growth |
| *POT1* | 3 | YIL160C peroxisomal 3-oxoacyl CoA thiolase |
| *FUS2* | 2.9 | YMR232W involved in cell fusion during mating |
| | 2.9 | YGL104C similarity to glucose transport proteins |
| | 2.9 | YHR140W hypothetical protein |
| | 2.8 | YOL015W weak similarity to YKR015c |
| *HXT16* | 2.8 | YJR158W hexose transporter |
| *MBR1* | 2.8 | YKL093W MBR1 protein precursor |
| *SNR54* | 2.8 | SNR54 snRNA |
| | 2.8 | YPL205C questionable ORF |
| | 2.8 | YLR252W questionable ORF |
| | 2.8 | YDR018C strong similarity to hypothetical protein YBR042c |
| | 2.8 | YLR251W sim. to peroxisomal rat membrane protein PMP22 |
| *GLG2* | 2.8 | YJL137C self-glucosylating initiator of glycogen synthesis |
| *DMC1* | 2.8 | YER179W meiosis-specific protein related to RecA and Rad51 |
| *PGM2* | 2.7 | YMR105C Phosphoglucomutase |
| *SNR76* | 2.7 | SNR76 snRNA |
| *PHO5* | 2.7 | YBR093C Acid phosphatase, repressible |
| | 2.7 | YKL031W hypothetical protein |
| | 2.7 | YLR324W strong similarity to YGR004w |
| *SHC1* | 2.7 | YER096W sporulation-specific homolog of csd4 |
| | 2.7 | YHL002c-a questionable ORF |
| | 2.6 | YJR098C weak similarity to *Bacillus licheniformis* esterase |
| *RTA1* | 2.6 | YGR213C involved in 7-aminocholesterol resistance |
| *MFα2* | 2.6 | YGL089C alpha mating factor |

| | | |
|---|---|---|
| *PHM7* | 2.6 | YOL084W similarity to *A. thaliana* hyp1 protein |
| | 2.6 | YOR173W strong similarity to YLR270w |
| | 2.6 | YGR043C strong similarity to transaldolase |
| *COS12* | 2.6 | YGL263W similarity to subtelomerically-encoded proteins |
| | 2.5 | YJR025c-a C-terminal part of YJR030c |
| *GSY2* | 2.5 | YLR258W Glycogen synthase |
| *PAU3* | 2.5 | YCR104W member of the seripauperin protein/gene family |
| | 2.5 | YBL098W similarity to kynurenine 3-monooxygenase (human) |
| *SNR75* | 2.5 | SNR75 snRNA |
| | 2.5 | YIL177C strong similarity to subtelomeric encoded proteins |
| *TES1* | 2.5 | YJR019C peroxisomal acyl-CoA thioesterase |
| *XYL2* | 2.5 | YLR070C strong similarity to sugar dehydrogenases |
| | 2.5 | YFL021c-a hypothetical protein |
| *CTT1* | 2.5 | YGR088W cytoplasmic catalase T |
| | 2.5 | YGL051W strong similarity to YAR033w protein |
| *MAL33* | 2.5 | YBR297W Maltose fermentation regulatory protein |
| | 2.5 | YFL030W similarity to several transaminases |
| | 2.5 | YMR191W hypothetical protein |
| | 2.5 | YHR054C weak similarity to YOR262w |
| *THI4* | 2.5 | YGR144W component of the thiamine biosynthetic pathway |
| | 2.5 | YKL071W weak similarity to *A. parasiticus* nor-1 protein |
| *AAD4* | 2.5 | YDL243C Hypothetical aryl-alcohol dehydrogenase |
| | 2.5 | YGR039W questionable ORF |
| | 2.5 | YOL037C questionable ORF |
| *SNR73* | 2.4 | SNR73 snRNA |
| *MAL32* | 2.4 | YBR299W Maltase (EC 3.2.1.20) |
| | 2.4 | YOL075C sim. to *A.gambiae* ATP-binding-cassette protein |
| *TIR1* | 2.4 | YER011W Cold-shock induced prot. of the Srp1p/Tip1p family |
| | 2.4 | YJL163C hypothetical protein |
| | 2.4 | YNL195C hypothetical protein |
| *GDH3* | 2.4 | YAL062W NADP-linked glutamate dehydrogenase |
| *PAU5* | 2.4 | YFL020C member of the seripauperin protein/gene family |
| | 2.3 | YJL068C strong similarity to human esterase D |
| | 2.3 | YDR428C hypothetical protein |
| | 2.3 | YER185W strong similarity to Rtm1p |
| | 2.3 | YER085C weak similarity to myosins |
| | 2.3 | YGL146C hypothetical protein |
| *FRM2* | 2.3 | YCL026C-A involved in the integration of lipid signaling |
| *WSC4* | 2.3 | YHL028W Putative integral membrane protein |
| *FCY22* | 2.3 | YER060w-A purine-cytosine permease |
| *CAT2* | 2.3 | YML042W Carnitine O-acetyltransferase |
| *DAL4* | 2.3 | YIR028W allantoin permease |
| *FIG2* | 2.3 | YCR089W predicted GPI-anchored cell wall protein |
| *DDR2* | 2.2 | YOL053C-A DNA Damage Responsive |
| *CYR1* | 2.2 | YJL005W adenylate cyclase |
| *PCK1* | 2.2 | YKR097W phosphoenolpyruvate carboxylkinase |

| | | |
|---|---|---|
| | 2.2 | YBL048W hypothetical protein |
| *CTR3* | 2.2 | YLR411W Copper Transporter |
| *AQY1* | 2.2 | YPR192W similarity to PM and water channel proteins |
| | 2.2 | YDL186W hypothetical protein |
| | 2.2 | YLR054C hypothetical protein |
| | 2.2 | YJL103C putative regulatory protein |
| | 2.2 | YGR149W hypothetical protein |
| *HXK1* | 2.2 | YFR053C Hexokinase I (PI) (also called Hexokinase A) |
| *ECM4* | 2.2 | YKR076W ExtraCellular Mutant |
| | 2.1 | YBR241C Probable sugar transport protein |
| | 2.1 | YMR187C hypothetical protein |
| *UBI4* | 2.1 | YLL039C ubiquitin |
| *TEP1* | 2.1 | YNL128W Similar to human tumor suppressor gene |
| *PMA2* | 2.1 | YPL036W plasma membrane ATPase |
| *FUN34* | 2.1 | YNR002C Putative transmembrane protein |
| *DAL3* | 2.1 | YIR032C ureidoglycolate hydrolase |
| | 2.1 | YER119C weak similarity to *E. herbicola* tyrosine permease |
| *YTP1* | 2.1 | YNL237W Yeast putative Transmembrane Protein |
| *MEP2* | 2.1 | YNL142W Ammonia transport protein |
| | 2.1 | YOL132W sim. to glycophospholipid-anchored surface protein |
| | 2.1 | YBL049W hypothetical protein |
| | 2.1 | YLR004C similarity to allantoate transport protein |
| | 2.1 | YHR209W similarity to hypothetical protein YER175c |
| | 2.1 | YIL015C-A strong similarity to hypothetical protein YIL102c |
| *SGA1* | 2.1 | YIL099W intracellular glucoamylase |
| | 2.1 | YIR041W similarity to members of the Srp1p/Tip1p family |
| | 2.1 | YJR160C strong similarity to Mal31p |
| *SRT1* | 2.1 | YMR101C similarity to YBR002c |
| | 2.1 | YMR325W similarity to members of the Srp1p/Tip1p family |
| | 2 | YOL128C strong similarity to protein kinase Mck1p |
| *RDN37-1* | 2 | RDN37-1 35S ribosomal RNA |
| | 2 | YDR516C strong similarity to glucokinase |
| *GAL4* | 2 | YPL248C zinc-finger transcription factor |

**Appendix Table 7.3.** Genes upregulated in *msi1*Δ cells. The average fold change result from two independent experiments is provided.

| Gene | Avg. Fold Δ | ORF and Description |
|---|---|---|
| | 55.7 | YGR111W weak similarity to mosquito carboxylesterase |
| | 24.3 | YOL162W strong similarity to hypothetical protein YIL166c |
| *SPO1* | 16.6 | YNL012W protein with similarity to phospholipase B |
| | 14.4 | YNR064C sim. to *R. capsulatus* 1-chloroalkane halidohydrolase |
| | 13 | YOL163W similarity to *P. putida* phthalate transporter |
| | 12.1 | YPR077C questionable ORF |
| | 12.1 | YJR078W sim. to mammalian indoleamine 2,3-dioxygenase |
| | 10.2 | YBL065W questionable ORF |
| *CRC1* | 8.9 | YOR100C similarity to mitochondrial carrier proteins |
| | 8.6 | YMR107W hypothetical protein |
| *DTR1* | 8 | YBR180W Probable resistance protein |
| | 7.7 | YOR387C strong similarity to YGL258w |
| | 7.7 | YBR178W questionable ORF |
| | 7 | YGL258W strong similarity to hypothetical protein YOR387c |
| *FIG1* | 7 | YBR040W integral membrane protein |
| | 6.7 | YMR322C similarity to YPL280w, YOR391c and YDR533c |
| | 6.7 | YMR118C strong similarity to succinate dehydrogenase |
| *THI11* | 6.5 | YJR156C Thiamine biosynthetic enzyme |
| | 6.3 | YLR149C hypothetical protein |
| | 6.3 | YGR066C similarity to hypothetical protein YBR105c |
| | 5.9 | YBR116C questionable ORF |
| *HUG1* | 5.9 | YML058w-a identified by SAGE |
| *TKL2* | 5.9 | YBR117C transketolase, homologous to tkl1 |
| *FDH1* | 5.9 | YOR388C Protein with similarity to formate dehydrogenases |
| *ADH2* | 5.7 | YMR303C alcohol dehydrogenase II |
| | 5.5 | YGL258W strong similarity to hypothetical protein YOR387c |
| | 5.1 | YBL070C questionable ORF |
| | 4.9 | YPL136W questionable ORF |
| *PTR2* | 4.6 | YKR093W Peptide transporter |
| | 4.4 | YHL006w-a questionable ORF |
| *YPS6* | 4.4 | YIR039C GPI-anchored aspartic protease |
| | 4.1 | YNR062C similarity to *H. influenzae* L-lactate permease |
| | 4 | YIR043C putative pseudogene |
| | 4 | YDR034W-B identified by SAGE expression analysis |
| *YPS5* | 4 | YGL259W GPI-anchored aspartic protease |
| | 4 | YNL034W nearly identical to YNL018c |
| *SPS19* | 3.9 | YNL202W peroxisomal 2,4-dienoyl-CoA reductase |
| *JEN1* | 3.9 | YKL217W carboxylic acid transporter protein homolog |
| | 3.7 | YJL037W strong similarity to hypothetical protein YJL038c |
| *THI2* | 3.7 | YBR240C Probable Zn-finger protein |

| | | |
|---|---|---|
| | 3.7 | YBR116C questionable ORF |
| *HSP12* | 3.7 | YFL014W 12 kDa heat shock protein |
| *GND2* | 3.7 | YGR256W 6-phosphogluconate dehydrogenase |
| | 3.6 | YGL104C similarity to glucose transport proteins |
| | 3.5 | YDL222C strong similarity to hypothetical protein |
| *GPH1* | 3.4 | YPR160W Glycogen phosphorylase |
| | 3.4 | YFL021c-a hypothetical protein |
| | 3.4 | YER119C weak similarity to E.herbicola tyrosine permease |
| | 3.4 | YNR073C similarity to *E. coli* D-mannonate oxidoreductase |
| *PRM1* | 3.4 | YNL279W hypothetical protein |
| | 3.4 | YJL045W similarity to succinate dehydrogenase flavoprotein |
| | 3.4 | YPR078C hypothetical protein |
| *ADY2* | 3.2 | YCR010C strong similarity to *Y. lipolytica* GPR1 protein |
| *SNR64* | 3.2 | SNR64 snRNA |
| *TIR1* | 3.2 | YER011W Cold-shock induced prot. of the Srp1p/Tip1p family |
| *THI4* | 3.2 | YGR144W component of the thiamine biosynthetic pathway |
| *STE3* | 3.2 | YKL178C a factor recptor |
| | 3.1 | YPR064W hypothetical protein |
| *COS12* | 3.1 | YGL263W similarity to subtelomerically-encoded proteins |
| *PHO5* | 3.1 | YBR093C Acid phosphatase, repressible |
| *FKS3* | 3.1 | YMR306W Protein with similarity to Gls1p and Gls2p |
| | 3.1 | YGL081W hypothetical protein |
| *SNR73* | 3 | SNR73 snRNA |
| *SNR76* | 3 | SNR76 snRNA |
| | 3 | YHL002c-a questionable ORF |
| *RTA1* | 2.9 | YGR213C involved in 7-aminocholesterol resistance |
| *GAL7* | 2.9 | YBR018C galactose-1-phosphate uridyl transferase |
| | 2.9 | TR(ACG)J tRNA-Arg |
| | 2.9 | YDL218W weak similarity to hypothetical protein YNR061c |
| | 2.9 | YKL031W hypothetical protein |
| *SMA1* | 2.9 | YPL027W hypothetical protein |
| | 2.8 | YHR140W hypothetical protein |
| | 2.8 | YGR131W strong similarity to Nce2p |
| *GAL10* | 2.8 | YBR019C UDP-glucose 4-epimerase |
| *MFα2* | 2.8 | YGL089C alpha mating factor |
| *PDC5* | 2.8 | YLR134W pyruvate decarboxylase |
| *SNR45* | 2.8 | SNR45 snRNA |
| *DMC1* | 2.8 | YER179W meiosis-specific protein related to RecA and Rad51 |
| *FCY22* | 2.8 | YER060w-A purine-cytosine permease |
| | 2.8 | YHR022C RAS-related protein |
| | 2.8 | YLR031W similarity to hypothetical protein YMR124w |
| | 2.7 | YLR252W questionable ORF |
| | 2.7 | YDL204W similarity to hypothetical protein YDR233c |
| | 2.7 | YBR241C Probable sugar transport protein |
| *DIT1* | 2.7 | YDR403W first enzyme in dityrosine synthesis |
| | 2.7 | YFL030W similarity to several transaminases |

| | | |
|---|---|---|
| | 2.7 | YGR039W questionable ORF |
| *GRE1* | 2.7 | YPL223C Induced by osmotic stress |
| *SNR74* | 2.6 | SNR74 snRNA |
| | 2.6 | YPL205C questionable ORF |
| | 2.6 | YGR149W hypothetical protein |
| *SGA1* | 2.6 | YIL099W intracellular glucoamylase |
| *FBP1* | 2.6 | YLR377C fructose-1,6-bisphosphatase |
| | 2.5 | YGL121C hypothetical protein |
| | 2.5 | YOL153C strong similarity to Cps1p |
| *SPS4* | 2.5 | YOR313C sporulation-specific protein |
| *MEP2* | 2.5 | YNL142W Ammonia transport protein |
| | 2.5 | YLR251W similarity to peroxisomal rat membrane protein |
| | 2.5 | YDR366C similarity to YOL106w and YER181c |
| *MAL33* | 2.5 | YBR297W Maltose fermentation regulatory protein |
| *KRE34* | 2.5 | YLR317W questionable ORF |
| | 2.5 | YGR201C similarity to translation elongation factor eEF1 |
| *SSE2* | 2.5 | YBR169C HSP70 family member, highly homologous to Sse1p |
| | 2.5 | YLR054C hypothetical protein |
| *ADR1* | 2.5 | YDR216W positive transcriptional regulator of ADH2 |
| | 2.5 | YFR018C sim. to human glutaminyl-peptide cyclotransferase |
| *PGU1* | 2.5 | YJR153W Endo-polygalacturonase |
| *SNR57* | 2.5 | SNR57 snRNA |
| | 2.4 | YDR428C hypothetical protein |
| *ARE1* | 2.4 | YCR048W Acyl-CoA cholesterol acyltransferase |
| *RHK1* | 2.3 | YBL082C Dol-P-Man dep. alpha(1-3) mannosyltransferase |
| *DUR3* | 2.3 | YHL016C Urea transporter |
| | 2.3 | YOR173W strong similarity to YLR270w |
| *XYL2* | 2.3 | YLR070C strong similarity to sugar dehydrogenases |
| *SNR13* | 2.3 | SNR13 snRNA |
| *FUN34* | 2.3 | YNR002C Putative transmembrane protein |
| | 2.3 | YJR025c-a C-terminal part of YJR030c |
| | 2.3 | YER152C weak similarity to *E. coli* hypothetical protein f470 |
| *BIO3* | 2.2 | YNR058W 7,8-diamino-pelargonic acid aminotransferase |
| | 2.2 | YOR083W weak similarity to YKR091w |
| | 2.2 | YCR062W similarity to Ytp1p protein |
| *OYE3* | 2.2 | YPL171C NAD(P)H dehydrogenase |
| *CSR1* | 2.2 | YLR380W weak similarity to SEC14 protein |
| *HXT9* | 2.2 | YJL219W High-affinity hexose transporter |
| | 2.2 | YIL071w-a questionable ORF |
| *PMA2* | 2.1 | YPL036W plasma membrane ATPase |
| | 2.1 | YCL033C Transcription regulator |
| *HSP26* | 2.1 | YBR072W heat shock protein 26 |
| | 2.1 | YGL051W strong similarity to YAR033w protein |
| *IMP2* | 2.1 | YIL154C transcription factor |
| | 2.1 | YMR034C weak similarity to YPR201w |
| *SNR54* | 2.1 | SNR54 snRNA |

| | | |
|---|---|---|
| *RRD1* | 2.1 | YIL153W Resistant to Rapamycin Deletion |
| *CHS7* | 2.1 | YHR142W weak similarity to cytochrome-c oxidases |
| *PAU3* | 2.1 | YCR104W member of the seripauperin gene family |
| *SMF1* | 2.1 | YOL122C localized to the plasma membrane |
| | 2.1 | YCR061W hypothetical protein |
| *BPL1* | 2.1 | YDL141W Biotin:apoprotein ligase |
| *AGA1* | 2.1 | YNR044W anchorage subunit of a-agglutinin |
| | 2.1 | YMR088C similarity to multidrug resistance proteins |
| | 2.1 | YER185W strong similarity to Rtm1p |
| | 2.1 | YOR292C similarity to human glomerulosclerosis protein |
| *PDR11* | 2 | YIL013C member of the ABC family of membrane transporters |
| *EMP46* | 2 | YLR080W strong similarity to Emp47p |
| *GDH3* | 2 | YAL062W NADP-linked glutamate dehydrogenase |

**Appendix Table 7.4.** Genes most significantly downregulated in *asf1*Δ cells. The average fold change result from two independent experiments is provided.

| Gene | Average Fold Δ | ORF and Description |
|---|---|---|
| | -13 | YDR524W-A identified by SAGE |
| | -8.3 | YGL102C questionable ORF |
| | -6.5 | YPR044C questionable ORF |
| | -6.3 | YPL142C questionable ORF |
| *BUD19* | -6.1 | YJL188C questionable ORF |
| | -4.9 | YOR277C questionable ORF |
| | -4.6 | YJLWDELTA19 Ty1 LTR |
| | -4.4 | YKL153W questionable ORF |
| | -4.4 | YDR417C questionable ORF |
| *PHO11* | -4.3 | YAR071W Acid phosphatase, secreted |
| | -4 | YPL197C questionable ORF |
| | -3.9 | YILCDELTA2 Ty2 LTR |
| *BUD28* | -3.6 | YLR062C questionable ORF |
| | -3.5 | YDL228C similarity to *A. klebsiana* glutamate dehydrogenase |
| | -3.4 | YNL174W hypothetical protein |
| | -3.4 | YERWDELTA18 Ty1 LTR |
| | -3.4 | YLR264c-a identified by SAGE |
| | -3.2 | YBR089W questionable ORF |
| *SNR31* | -3.2 | SNR31 snRNA |
| | -3.1 | YLL044W questionable ORF |
| | -3 | YLR198C questionable ORF |
| | -2.9 | YERWDELTA18 Ty1 LTR |
| | -2.7 | YDR209C questionable ORF |
| *CWH36* | -2.7 | YCL007C Calcofluor White Hypersensitivity |
| *ABP140* | -2.6 | YOR239W hypothetical protein |
| | -2.5 | SNR45 snRNA |
| | -2.5 | YOR385W strong similarity to hypothetical protein YMR316w |
| *APL5* | -2.5 | YPL195W delta-like subunit of the yeast AP-3 |
| *TDH1* | -2.5 | YJL052W Glyceraldehyde-3-phosphate dehydrogenase 1 |
| | -2.5 | YDR524c-a identified by SAGE |
| *RPS23B* | -2.5 | YPR132W Ribosomal protein S23B (S28B) (rp37) (YS14) |
| *RPL24A* | -2.5 | YGL031C Ribosomal protein L24A (rp29) (YL21) (L30A) |
| *TPI1* | -2.5 | YDR050C triosephosphate isomerase |
| *TDH3* | -2.5 | YGR192C Glyceraldehyde-3-phosphate dehydrogenase 3 |
| | -2.2 | YOL014W hypothetical protein |
| *RDN18-1* | -2.2 | RDN18-1 18S ribosomal RNA |
| *RPL30* | -2.2 | YGL030W Large ribosomal subunit protein L30 (L32) |
| *RPL1A* | -2.2 | YPL220W Ribosomal protein L1A |
| *ADH1* | -2.2 | YOL086C Alcohol dehydrogenase |

| | | |
|---|---|---|
| *RPP0* | -2.2 | YLR340W 60S ribosomal protein P0 (A0) (L10E) |
| *RPL10* | -2.3 | YLR075W Ribosomal protein L10 |
| *ENO1* | -2.3 | YGR254W enolase I |
| *RPP2B* | -2.3 | YDR382W Ribosomal protein P2B (YP2beta) (L45) |
| *PSA1* | -2.3 | YDL055C mannose-1-phosphate guanyltransferase, |
| *PGK1* | -2.3 | YCR012W 3-phosphoglycerate kinase |
| *RPL11A* | -2.3 | YPR102C Ribosomal protein L11A (L16A) (rp39A) (YL22) |
| *TEF1* | -2.3 | YPR080W translational elongation factor EF-1 alpha |
| *ASC1* | -2.3 | YMR116C WD repeat protein (G-beta like protein) |
| *CMK2* | -2.3 | YOL016C Calmodulin-dependent protein kinase |
| *RPS31* | -2.3 | YLR167W Ribosomal protein S31 (S37) (YS24) |
| | -2.3 | YHR063w-a questionable ORF |
| *CCW12* | -2.3 | YLR110C strong similarity to Flo1p |
| *RPP1A* | -2.2 | YDL081C Acidic ribosomal protein P1A (YP1alpha) (A1) |
| *FBA1* | -2.2 | YKL060C aldolase |
| *GPM1* | -2.2 | YKL152C Phosphoglycerate mutase |
| *RPL31A* | -2.2 | YDL075W Ribosomal protein L31A (L34A) (YL28) |
| *RPS9B* | -2.2 | YBR189W Ribosomal protein S9B (S13) (rp21) (YS11) |
| *RPS12* | -2.2 | YOR369C 40S ribosomal protein S12 |
| *RPL26A* | -2.2 | YLR344W Ribosomal protein L26A (L33A) (YL33) |
| *RPS21A* | -2.2 | YKR057W Ribosomal protein S21A (S26A) (YS25) |
| *TDH2* | -2.2 | YJR009C glyceraldehyde 3-phosphate dehydrogenase |
| *ERG11* | -2.2 | YHR007C cytochrome P450 lanosterol 14a-demethylase |
| *RPS2* | -2.2 | YGL123W Ribosomal protein S2 (S4) (rp12) (YS5) |
| *RPL2A* | -2.2 | YFR031C-A Ribosomal protein L2A (L5A) (rp8) (YL6) |
| *ASC1* | -2.2 | YMR116C WD repeat protein (G-beta like protein) |
| *RDN37-1* | -2.2 | RDN37-1 35S ribosomal RNA |
| | -2.2 | YBL094C questionable ORF |
| | -2.1 | YOR169C questionable ORF |
| | -2.1 | YNL217W weak similarity to E.coli protein |
| | -2.1 | YBL077W questionable ORF |
| *RPS10A* | -2.1 | YOR293W Ribosomal protein S10A |
| *BDF1* | -2.1 | YLR399C Bdf1p contains two bromodomains |
| *CBF5* | -2.1 | YLR175W major low affinity 55 kDa Centromere binding prot. |
| *PDC1* | -2.1 | YLR044C pyruvate decarboxylase |
| *RPL14A* | -2.1 | YKL006W Ribosomal protein L14A |
| | -2.1 | YDR524c-a identified by SAGE |
| *RPL4A* | -2.1 | YBR031W Ribosomal protein L4A (L2A) (rp2) (YL2) |
| | -2.1 | YOR102W questionable ORF |
| *RPL20A* | -2.1 | YMR242C Ribosomal protein L20A (L18A) |
| *YEF3* | -2.1 | YLR249W EF-3 (translational elongation factor 3) |
| *RPL16A* | -2.1 | YIL133C Ribosomal protein L16A (L21A) (rp22) (YL15) |
| *ENO2* | -2.1 | YHR174W enolase |
| *RPS9B* | -2.1 | YBR189W Ribosomal protein S9B (S13) (rp21) (YS11) |
| *RPS8A* | -2.1 | YBL072C Ribosomal protein S8A (S14A) (rp19) (YS9) |
| | -2.1 | YPL238C questionable ORF |

| | | |
|---|---|---|
| | -2.1 | YOR309C questionable ORF |
| *RPS17A* | -2.1 | YML024W Ribosomal protein S17A (rp51A) |
| *RPL24B* | -2.1 | YGR148C Ribosomal protein L24B (rp29) (YL21) (L30B) |
| *RPL9A* | -2.1 | YGL147C Ribosomal protein L9A (L8A) (rp24) (YL11) |
| *ACT1* | -2.1 | YFL039C Actin |
| *RPS3* | -2.1 | YNL178W Ribosomal protein S3 (rp13) (YS3) |
| *RPL23A* | -2.1 | YBL087C Ribosomal protein L23A (L17aA) (YL32) |
| *RPS21B* | -2.1 | YJL136C Ribosomal protein S21B (S26B) (YS25) |
| | -2.1 | YERWDELTA18 Ty1 LTR |
| *RPL34B* | -2.1 | YIL052C Ribosomal protein L34B |
| *UTP8* | -2.1 | YGR128C hypothetical protein |
| *RPL11A* | -2.1 | YPR102C Ribosomal protein L11A (L16A) (rp39A) (YL22) |
| *RPS6A* | -2.1 | YPL090C Ribosomal protein S6A (S10A) (rp9) (YS4) |
| *RPS10B* | -2.1 | YMR230W Ribosomal protein S10B |
| | -2.1 | YKL056C similarity to human IgE-dependent histamine-factor |
| | -2.1 | YNL043C questionable ORF |
| *SSB2* | -2.1 | YNL209W Heat shock protein of HSP70 family |
| *RPS22A* | -2.1 | YJL190C Ribosomal protein S22A (S24A) (rp50) (YS22) |
| | -2.1 | YERWDELTA18 Ty1 LTR |
| *RPS27B* | -2.1 | YHR021C 40S Ribosomal protein S27B (rp61) (YS20) |
| *RPL28* | -2.1 | YGL103W Ribosomal protein L28 (L29) (rp44) (YL24) |
| | -2.1 | YDR154C questionable ORF |
| *RPL4B* | -2.1 | YDR012W Ribosomal protein L4B (L2B) (rp2) (YL2) |
| *RPP1B* | -2.1 | YDL130W Ribosomal protein P1B (L44 ) (YP1beta) (Ax) |
| *PGI1* | -2.1 | YBR196C Glucose-6-phosphate isomerase |
| *RPL5* | -2.1 | YPL131W Ribosomal protein L5 (L1a)(YL3) |
| *EFT1* | -2.1 | YOR133W translation elongation factor 2 (EF-2) |
| *RPL18B* | -2.1 | YNL301C Ribosomal protein L18B (rp28B) |
| *RPS7A* | -2 | YOR096W Ribosomal protein S7A (rp30) |
| *RPL14A* | -2 | YKL006W Ribosomal protein L14A |
| *RPL8B* | -2 | YLL045C Ribosomal protein L8B (L4B) (rp6) (YL5) |
| *RPS4A* | -2 | YJR145C Ribosomal protein S4A (YS6) (rp5) (S7A) |
| *YCK1* | -2 | YHR135C membrane-bound casein kinase I homolog |
| *RPL43A* | -2 | YPR043W Ribosomal protein L43A |
| *RPS1A* | -2 | YLR441C Ribosomal protein S1A (rp10A) |
| *RPL17B* | -2 | YJL177W Ribosomal protein L17B (L20B) (YL17) |

**Appendix Table 7.5.** Genes downregulated in *cac1*Δ cells. The average fold change result from two independent experiments is provided.

| Gene | Avg. Fold Δ | ORF and Description |
|---|---|---|
| *DIA1* | -3.7 | YMR316W similarity to YOR385w and YNL165w |
| *NOP16* | -3.1 | YER002W similarity to chicken microfibril-associated protein |
| *BUR6* | -3.1 | YER159C Transcriptional regulator |
| *PCL5* | -3 | YHR071W G1/S cyclin (weak) |
| *YKE2* | -2.9 | YLR200W Component of Yeast Actin Binding Complex |
| | -2.9 | YMR316C-A questionable ORF |
| | -2.8 | YKL082C weak similarity to *C. elegans* hypothetical protein |
| *FIT2* | -2.8 | YOR382W hypothetical protein |
| | -2.8 | YPR143W hypothetical protein |
| *CPA2* | -2.7 | YJR109C carbamyl phosphate synthetase |
| | -2.7 | YKR100C cause growth inhibition when overexpressed |
| *FYV14* | -2.7 | YDL213C has an RNA recognition domain in the N-terminus |
| *RRP3* | -2.7 | YHR065C DEAD box gene homologous to eIF-4A |
| *RRP7* | -2.7 | YCL031C pre-rRNA processing and ribosome assembly |
| | -2.7 | YML053C hypothetical protein |
| *ERV1* | -2.6 | YGR029W essential for mito. biogenesis and viability |
| *SRB7* | -2.6 | YDR308C RNA polymerase II holoenzyme component |
| | -2.6 | YLR003C hypothetical protein |
| *DID2* | -2.6 | YKR035W-A RAD52 Inhibitor (Fifty Two Inhibitor) |
| *BDF1* | -2.6 | YLR399C Bdf1p contains two bromodomains |
| | -2.6 | YOR289W similarity to *C. elegans* hypothetical protein |
| *VPS28* | -2.5 | YPL065W protein involved in transport |
| *RSA1* | -2.5 | YPL193W weak similarity to human centromere protein E |
| *IST3* | -2.5 | YGR029W Protein essential for mitochondrial biogenesis |
| | -2.5 | YIL161W hypothetical protein |
| *GCV1* | -2.5 | YDR019C glycine cleavage T protein |
| *ERV1* | -2.5 | YGR029W Protein essential for mitochondrial biogenesis |
| *RRP1* | -2.5 | YDR087C involved in processing rRNA precursor species |
| | -2.5 | YGR130C weak similarity to myosin heavy chain proteins |
| *KRR1* | -2.5 | YCL059C similarity to human Rev interacting protein Rip-1 |
| | -2.5 | YCR016W hypothetical protein |
| *VPS20* | -2.5 | YMR077C similarity to SNF7 protein |
| | -2.5 | YNL129W similarity to *M. pneumoniae* uridine kinase udk |
| | -2.5 | YBR056w-a identified by SAGE |
| *YHC1* | -2.5 | YLR298C U1 snRNP protein required for pre-mRNA splicing |
| *CDC31* | -2.5 | YOR257W calcium-binding protein |
| *NBP2* | -2.5 | YDR162C Nap1p-binding protein |
| | -2.4 | YIL127C weak similarity to Smy2p |
| *IST3* | -2.4 | YIR005W similarity to RNA-binding proteins |
| *MSL1* | -2.4 | YIR009W encodes YU2B, a component of yeast U2 snRNP |

| | | |
|---|---|---|
| *CBP6* | -2.4 | YBR120C Translational activator of COB mRNA |
| *VID31* | -2.4 | YKL054C similarity to glutenin |
| *PHM8* | -2.4 | YER037W strong similarity to hypothetical protein YGL224c |
| | -2.4 | YER130C similarity to Msn2p and weak similarity to Msn4p |
| | -2.4 | YLR168C involved in intramitochondrial protein sorting |
| | -2.4 | YGL117W hypothetical protein |
| | -2.4 | YCR087C-A nucleic acid-binding protein |
| *RRP8* | -2.4 | YDR083W similarity to hypothetical *S. pombe* protein |
| | -2.4 | YJL184W hypothetical protein |
| *RSM18* | -2.3 | YER050C hypothetical protein |
| *GFD1* | -2.3 | YMR255W hypothetical protein |
| *MTD1* | -2.3 | YKR080W 5,10-methylenetetrahydrafolate dehydrogenase |
| *BTN2* | -2.3 | YGR142W similarity to hypothetical protein YPR158w |
| *YRB2* | -2.3 | YIL063C nuclear protein, interacts with Gsp1p and Crm1p |
| *DRE2* | -2.3 | YKR071C weak similarity to *C. elegans* hypothetical protein |
| *CWC21* | -2.3 | YDR482C hypothetical protein |
| *RPC34* | -2.3 | YNR003C 34-kDa subunit of RNA polymerase III (C) |
| *RLP7* | -2.3 | YNL002C Significant sequence similarity to RPL7B |
| *CNS1* | -2.3 | YBR155W Stress-inducible riboflavin biosynthetic protein |
| *RPA43* | -2.3 | YOR340C DNA-dependent RNA polymerase I subunit A43 |
| *FPR3* | -2.3 | YML074C Prolyl cis-trans isomerase |
| *CMK2* | -2.3 | YOL016C Calmodulin-dependent protein kinase |
| *PET123* | -2.3 | YOR158W Mitochondrial ribosomal protein of small subunit |
| *LOC1* | -2.2 | YFR001W weak similarity to rabbit triadin Spp41p |
| *RPN12* | -2.2 | YFR052W cytoplasmic 32 - 34 kDa protein |
| *BNI5* | -2.2 | YNL166C hypothetical protein |
| *GPX2* | -2.2 | YBR244W Probable glutathione peroxidase (EC 1.11.1.9) |
| *SAS10* | -2.2 | YDL153C Something About Silencing 10 |
| *RLP24* | -2.2 | YLR009W similarity to ribosomal protein L24.e.B |
| *RPB5* | -2.2 | YBR154C 25-kDa RNA polymerase subunit |
| *ISU2* | -2.2 | YOR226C NifU-like protein A |
| *NGG1* | -2.2 | YDR176W transcription factor |
| | -2.2 | YOR006C similarity to *M. jannaschii* hypothetical protein |
| *KRI1* | -2.2 | YNL308C similarity to *S. pombe* hypothetical proteins |
| *RTT102* | -2.1 | YGR275W hypothetical protein |
| | -2.1 | YDR152W weak similarity to *C. elegans* hypothetical protein |
| *IOC4* | -2.1 | YMR044W hypothetical protein |
| | -2.1 | YOR385W similarity to hypothetical protein YMR316w |
| *SDC1* | -2.1 | YDR469W hypothetical protein |
| | -2.1 | YKR007W weak similarity to Streptococcus protein M5 |
| *NOC3* | -2.1 | YLR002C similarity to hypothetical *C. elegans* protein |
| | -2.1 | YDR248C similarity to *E. coli* thermoresistant gluconokinase |
| | -2.1 | YKL083W questionable ORF |
| *KTI12* | -2.1 | YKL110C involved in resistance to *K. lactis* killer toxin |
| *RIB5* | -2.1 | YBR256C Riboflavin synthase alpha-chain |
| *TRI1* | -2.1 | YMR233W strong similarity to YOR295w |

| | | |
|---|---|---|
| *ASE1* | -2.1 | YOR058C encodes component of the spindle midzone |
| | -2.1 | YOR004W weak similarity to hypothetical protein YDR339c |
| *FYV6* | -2.1 | YNL133C hypothetical protein |
| *VTI1* | -2.1 | YMR197C a v-SNARE that interacts with two t-SNARES |
| | -2.1 | YIR010W hypothetical protein |
| *HPC2* | -2.1 | YBR215W highly charged, basic protein |
| *SMB1* | -2 | YER029C Associated with U1 snRNP |
| *CWC15* | -2 | YDR163W weak similarity to *S. pombe* hypothetical protein |
| *RPF1* | -2 | YHR088W similarity to hypothetical protein YNL075w |
| *INO4* | -2 | YOL108C TF involved in activation of phospholipid synth. |
| | -2 | YDL173W hypothetical protein |
| *TAF65* | -2 | YML114C hypothetical protein |
| *IWS1* | -2 | YPR133C similarity to *C. elegans* hypothetical protein |
| *RPB9* | -2 | YGL070C RNA polymerase II subunit |
| *ERP1* | | YAR002C-A p24 protein involved in membrane trafficking |

**Appendix Table 7.6.** Genes downregulated in *msi1*Δ cells. The average fold change result from two independent experiments is provided.

| Gene | Avg. Fold Δ | ORF and Description |
|---|---|---|
| *RPA34* | -5.1 | YJL148W RNA polymerase I subunit, not shared (A34.5) |
| *LSM1* | -4.1 | YJL124C Like Sm-B protein |
| *CPA2* | -3.9 | YJR109C carbamyl phosphate synthetase |
| | -3.7 | YJL122W weak similarity to dog-fish transition protein S2 |
| *ECM1* | -3.5 | YAL059W ExtraCellular Mutant |
| *TIM54* | -3.5 | YJL054W Translocase for the insertion of proteins into the mito. |
| | -3.4 | YJR014W strong similarity to *S. pombe* hypothetical protein |
| *CYC1* | -3.2 | YJR048W iso-1-cytochrome c |
| *CAP2* | -3.2 | YIL034C beta subunit of capping protein |
| *MNN11* | -3.1 | YJL183W related to Mnn10p |
| | -3.1 | YJL055W similarity to *R. fascians* hypothetical protein 6 |
| *VMA22* | -3.1 | YHR060W required for V-ATPase activity |
| *RNA15* | -3.1 | YGL044C component of the cleavage and polyadenylation factor |
| *GCD14* | -3 | YJL125C translational repressor of GCN4 |
| *HAP4* | -2.9 | YKL109W transcriptional activator protein of CYC1 |
| | -2.9 | YLR003C hypothetical protein |
| *VID31* | -2.9 | YKL054C similarity to glutenin and Snf5p |
| *SDS22* | -2.9 | YKL193C Interacts with & may be a positive regulator of GLC7 |
| *ILV3* | -2.9 | YJR016C dihydroxyacid dehydratase |
| *STE18* | -2.9 | YJR086W subunit of G protein coupled to mating factor receptors |
| | -2.9 | YGR122W hypothetical protein |
| *CCR4* | -2.8 | YAL021C 95 kDa containng leucine rich tandem repeats |
| | -2.8 | YJL145W weak similarity to T.pacificus retinal-binding protein |
| *RFA1* | -2.8 | YAR007C 69 kDa subunit of the heterotrimeric RPA |
| *OSM1* | -2.8 | YJR051W osmotic growth protein |
| | -2.7 | YJR084W weak similarity to *S. pombe* hypothetical protein |
| *RRP7* | -2.7 | YCL031C pre-rRNA processing and ribosome assembly |
| | -2.7 | YJL178C hypothetical protein |
| *PHO86* | -2.7 | YJL117W Putative inorganic phosphate transporter |
| *ISY1* | -2.7 | YJR050W Interacts with Syf1p |
| | -2.6 | YJL010C weak similarity to *C. elegans* hypothetical protein |
| *RSA1* | -2.6 | YPL193W weak similarity to human centromere protein E |
| | -2.6 | YOR289W similarity to *C. elegans* hypothetical protein |
| | -2.6 | YML053C hypothetical protein |
| | -2.6 | YJL200C strong similarity to aconitate hydratase |
| | -2.6 | YJR056C hypothetical protein |
| *PCL5* | -2.6 | YHR071W G1/S cyclin (weak) |
| *ARP4* | -2.6 | YJL081C 54.8 kDa actin-related protein |
| *NOP16* | -2.6 | YER002W similarity to chicken microfibril-associated protein |

| | | |
|---|---|---|
| | -2.5 | YKL082C weak similarity to *C. elegans* hypothetical protein |
| *RLP7* | -2.5 | YNL002C Significant sequence similarity to RPL7B |
| *SSC1* | -2.5 | YJR045C Mito. matrix protein involved in protein import\ |
| *DRE3* | -2.5 | YIL003W sim. to Nbp35p and human nucleotide-binding protein |
| *SRP21* | -2.5 | YKL122C component of signal recognition particle |
| *FBP26* | -2.5 | YJL155C Fructose-2,6-bisphosphatase |
| *ARG1* | -2.5 | YOL058W arginosuccinate synthetase |
| *RPL17B* | -2.5 | YJL177W Ribosomal protein L17B (L20B) (YL17) |
| *MAM33* | -2.5 | YIL070C mitochondrial acidic matrix protein |
| *BET1* | -2.5 | YIL004C Synaptobrevin (t-SNARE) homolog |
| *CYS4* | -2.5 | YGR155W Cystathionine beta-synthase |
| *TDH1* | -2.5 | YJL052W Glyceraldehyde-3-phosphate dehydrogenase 1 |
| *HCA4* | -2.5 | YJL033W putative RNA helicase |
| *ADE1* | -2.4 | YAR015W phosphoribosyl amino imidazolesuccinocarbozamide |
| *TIF3* | -2.4 | YPR163C Translation initiation factor eIF-4B |
| | -2.4 | YGR058W similarity to mouse calcium-binding protein |
| *NIT2* | -2.4 | YJL126W Nit2 nitrilase |
| *MNN5* | -2.4 | YJL186W putative mannosyltransferase |
| *OPT1* | -2.4 | YJL212C strong similarity to *S. pombe* isp4 protein |
| | -2.4 | YJL123C similarity to *D. melanogaster* troponin T |
| *RPL39* | -2.4 | YJL189W Ribosomal protein L39 (L46) (YL40) |
| *SER33* | -2.4 | YIL074C similarity to *E. coli* phosphoglycerate dehydrogenase |
| *ECM13* | -2.3 | YBL043W ExtraCellular Mutant |
| *CCT3* | -2.3 | YJL014W Cytoplasmic chaperonin subunit gamma |
| *BDF1* | -2.3 | YLR399C Bdf1p contains two bromodomains |
| *CCT7* | -2.3 | YJL111W Chaperonin Containing T-complex subunit seven |
| *ARA1* | -2.3 | YBR149W D-arabinose dehydrogenase |
| *BUR6* | -2.3 | YER159C Transcriptional regulator |
| *TFG1* | -2.3 | YGR186W Transcription factor TFIIF large subunit |
| *RPB4* | -2.3 | YJL140W fourth-largest subunit of RNA polymerase II |
| *RPC34* | -2.3 | YNR003C 34-kDa subunit of RNA polymerase III (C) |
| *RIB5* | -2.2 | YBR256C Riboflavin synthase alpha-chain |
| | -2.2 | YKL099C similarity to *C. elegans* hypothetical proteins |
| *RCS1* | -2.2 | YGL071W Putative TF that binds the PyPuCACCCPu consensus |
| *YHC1* | -2.2 | YLR298C U1 snRNP protein required for pre-mRNA splicing |
| | -2.2 | YPR143W hypothetical protein |
| *RLP24* | -2.2 | YLR009W similarity to ribosomal protein L24.e.B |
| *RRP3* | -2.2 | YHR065C RRP3 is a DEAD box gene homologous to eIF-4a |
| *SME1* | -2.1 | YOR159C homologue of human E core protein |
| *KRR1* | -2.1 | YCL059C similarity to human Rev interacting protein Rip-1 |
| *RPL1* | -2.1 | YJL177W Ribosomal protein L17B (L20B) (YL17) |
| | -2.1 | YJL217W hypothetical protein |
| *GFD1* | -2.1 | YMR255W hypothetical protein |
| | -2.1 | YPL260W hypothetical protein |
| *ANC1* | -2.1 | YPL129W TFIIF subunit (transcription initiation factor), 30 kD |
| | -2.1 | YKR060W hypothetical protein |

| | | |
|---|---|---|
| *RPA43* | -2.1 | YOR340C DNA-dependent RNA polymerase I subunit A43 |
| *SSY5* | -2.1 | YJL156C sensitive to sulfonylurea herbicides |
| *APM1* | -2.1 | YPL259C subunit of the clathrin-associated protein complex |
| *KRI1* | -2.1 | YNL308C sim. to *S. pombe* and *C. elegans* hypothetical proteins |
| *RPL17B* | -2.1 | YJL177W Ribosomal protein L17B (L20B) (YL17) |
| | -2.1 | YJR088C similarity to S. pombe hypothetical protein |
| *YVH1* | -2.1 | YIR026C nitrogen starvation-induced protein phosphatase |
| *MRT4* | -2.1 | YKL009W mRNA turnover 4 |
| *PRP11* | -2.1 | YDL043C snRNA-associated protein |
| *VPS20* | -2.1 | YMR077C similarity to SNF7 protein |
| *DRS1* | -2 | YLL008W putative ATP dependent RNA helicase |
| *NOP14* | -2 | YDL148C similarity to human mRNA clone RES4-25 |
| | -2 | YJR013W similarity to *C. elegans* B0491.1 protein |

**Appendix II:**

**Microarray Data for *apc10Δ* cells**

**Appendix Table 8.1.** Genes most significantly upregulated in *apc10Δ* cells. The results of two independent experiments are provided.

| Gene/ORF | Fold Δ1 | Fold Δ2 | Description |
|---|---|---|---|
| SSA2 | 83.9 | 104.7 | Member of 70 kDa heat shock protein family |
| *CYS3* | 20.5 | 56.1 | Cystathionine gamma-lyase |
| YMR318C | 20.3 | 23.8 | Strong similarity to alcohol-dehydrogenase |
| *IMD4 EX2* | 17.7 | 15.4 | Strong similarity to IMP dehydrogenases |
| *SAM1* | 15.1 | 14.7 | S-adenosylmethionine synthetase |
| *FIG2* | 14.9 | 10.5 | Predicted GPI-anchored cell wall protein |
| *HXT4* | 14.4 | 13.2 | Hexose transporter |
| *PDR12* | 13.8 | 18.7 | Multidrug resistance transporter |
| *EXG2* | 13.7 | 10.7 | Exo-1,3-b-glucanase |
| YOR049C | 12.7 | 20.8 | Similarity to YER185w, Rta1p |
| YMR102C | 12.1 | 8.8 | Strong similarity to YKL121w |
| YFR018C | 11.7 | 12 | Sim. to glutaminyl-peptide cyclotransferase |
| *KTR2* | 11.5 | 10 | Putative mannosyltransferase\; type 2 membrane prot. |
| *MKC7* | 11.4 | 13.4 | Aspartyl protease related to Yap3p |
| *GND1* | 11.2 | 11.3 | Phosphogluconate Dehydrogenase (Decarboxylating) |
| *PTR2* | 10.9 | 12.5 | Peptide transporter |
| YBL081W | 10.8 | 9 | Hypothetical protein |
| *ERG4* | 10.5 | 10 | Sterol C-24 reductase |
| *FET3* | 10.3 | 11.5 | Multicopper oxidase |
| YGL157W | 10.2 | 10.6 | Similarity to *V. vinifera* dihydroflavonol 4-reductase |
| YLR413W | 10 | 12.9 | Strong similarity to YKL187c |
| *PDR5* | 9.9 | 8.6 | Multidrug resistance transporter |
| *SCP160* | 9.8 | 2.2 | May be required during cell division |
| YAR075W | 9.6 | 2.1 | Strong similarity to IMP dehydrogenases |
| *BDH1* | 9.3 | 1.7 | (2R,3R)-2,3-butanediol dehydrogenase |
| *UTR2* | 9.2 | 5.8 | Weak similarity to *Bacillus* 1,3-1,4-beta-glucanase |
| *VTH2* | 9.2 | 7.5 | Strong similarity to Pep1p |
| *TUB3 EX2* | 9.1 | 10.7 | Alpha-tubulin |
| *COQ2* | 8.9 | 11.1 | Para hydroxybenzoate: polyprenyl transferase |
| *MYO4* | 8.7 | 2 | Myosin |
| *PHO3* | 8.5 | 9.6 | Acid phosphatase, constitutive |
| *ASN2* | 8.4 | 8.7 | Asparagine synthetase |
| *DPH2* | 8.4 | 2 | Diptheria toxin resistance protein |
| *GDH3* | 8.2 | 3.2 | NADP-linked glutamate dehydrogenase |
| *AGA1* | 8.1 | 11.8 | Anchorage subunit of a-agglutinin |
| *TOR1* | 8.1 | 3.6 | Involved in cell cycle signaling and meiosis |
| *HXT1* | 8 | 6.3 | High-affinity hexose (glucose) transporter |
| *ABP1* | 7.9 | 7.2 | Actin binding protein |
| *HXT2* | 7.9 | 7.3 | High affinity hexose transporter-2 |

| | | | |
|---|---|---|---|
| YNR014W | 7.9 | 7.3 | Weak similarity to hypothetical protein YMR206w |
| *ARG1* | 7.8 | 9 | Arginosuccinate synthetase |
| YKL044W | 7.8 | 8.3 | Hypothetical protein |
| *THR1* | 7.6 | 8 | Homoserine kinase |
| *GUA1* | 7.5 | 7 | GMP synthase |
| *PGM1* | 7.5 | 6.1 | Phosphoglucomutase, minor isoform |
| *PIR1* | 7.5 | 3.6 | Protein containing tandem internal repeats |
| *YSR3* | 7.5 | 5.1 | DHS-1-P phosphatase |
| *MUP3* | 7.4 | 7.3 | Methionine uptake |
| *PMU1* | 7.4 | 6.3 | Phospo-mutase homolog |
| *SRL1* | 7.4 | 7.3 | Suppressor of rad53 lethality |
| *AAT1* | 7.3 | 5.4 | Aspartate aminotransferase, mitochondrial |
| *KRE11* | 7.3 | 6.9 | Involved in biosynthetic pathway for cell wall |
| *PRM10* | 7.2 | 6.7 | Pheromone-regulated membrane protein |
| *YVH1* | 7.2 | 8.4 | Nitrogen starvation-induced protein phosphatase |
| *ADO1* | 7 | 4.5 | Adenosine kinase |
| *MEP3* | 7 | 10.6 | NH4+ transporter, highly similar to Mep1p and 2p |
| *MRPL7* | 7 | 7.7 | Mitochondrial ribosomal protein MRPL7 (YmL7) |
| *REC107 EX2* | 7 | 3.9 | Meiotic recombination protein |
| YLR187W | 7 | 8.3 | Similarity to hypothetical protein YNL278w |
| *GPH1* | 6.9 | 6.4 | Glycogen phosphorylase |
| *MRK1 EX1* | 6.9 | 8.9 | MDS1 related protein kinase |
| *PAN6* | 6.9 | 6.9 | Similarity to *E. coli* pantothenate synthetase |
| *TEF4 EX2* | 6.9 | 3.3 | Translation elongation factor EF-1gamma |
| *SSA1* | 6.7 | 2.2 | Heat shock protein of HSP70 family, cytoplasmic |
| YPR114W | 6.7 | 8.2 | Similarity to YJR116w |
| *FIT3* | 6.6 | 7.6 | Weak similarity to L.mexicana secreted acid |
| YAR068W | 6.6 | 4.1 | Potential membrane protein |
| *CLN2* | 6.5 | 8.4 | G(sub)1 cyclin |
| *MRS3* | 6.5 | 4.6 | Mitochondrial carrier protein |
| YDL189W | 6.5 | 4.9 | Hypothetical protein |
| YLR177W | 6.5 | 7 | Similarity to suppressor protein Psp5p |
| *FIT2* | 6.4 | 6.8 | Hypothetical protein |
| YFL054C | 6.4 | 6.3 | Similarity to channel proteins |
| *ATF2* | 6.3 | 8.2 | Alcohol acetyltransferase |
| *MTO1* | 6.2 | 3.5 | Mitochondrial Translation Optimization |
| *SWD3* | 6.2 | 8.6 | Likely involved in chromatin remodeling |
| *FCY21* | 6.1 | 5.7 | Purine-cytosine permease |
| *LOT5* | 6.1 | 4 | Hypothetical protein |
| *MUP1* | 6.1 | 6.4 | High affinity methionine permease |
| *ARE1* | 6 | 7.9 | Acyl-CoA cholesterol acyltransferase |
| *MET2* | 6 | 7.4 | Homoserine O-trans-acetylase |
| *SPC25* | 6 | 5.5 | Component of spindle pole |
| YIL121W | 6 | 7.2 | Similarity to antibiotic resistance proteins |
| YNL045W | 6 | 5.6 | Strong similarity to human leukotriene-A4 hydrolase |
| YOL161C | 6 | 8.2 | Similarity to members of the Srp1p/Tip1p family |

| | | | |
|---|---|---|---|
| *CDC9* | 5.9 | 6 | DNA ligase |
| *ENB1* | 5.9 | 6.7 | Similarity to subtelomeric encoded proteins |
| *PAC11* | 5.8 | 5.4 | Protein required in the absence of Cin8p |
| YJL069C | 5.8 | 5.3 | Similarity to C.elegans hypothetical protein |
| YPL245W | 5.8 | 7.9 | Weak similarity to human mutL protein homolog |
| *FRS2* | 5.7 | 4.9 | Phenylalanyl-tRNA synthetase, beta subunit |
| *LAP4* | 5.7 | 3.4 | Vacuolar aminopeptidase ysc1 |
| *RAX2* | 5.7 | 10.1 | Involved in the maintenance of bipolar pattern |
| *RHK1* | 5.7 | 4.6 | Alpha(1-3) mannosyltransferase |
| YNR065C | 5.7 | 5.9 | Strong similarity to YJL222w, YIL173w and Pep1p |
| YOR108W | 5.7 | 5.6 | Putative isoform of Leu4p |
| YPL137C | 5.7 | 3.8 | Similarity to microtubule-interacting protein Mhp1p |
| *ELO1* | 5.6 | 2.7 | Elongation enzyme 1 |
| *GRH1* | 5.6 | 5.8 | Weak similarity to hypothetical *S. pombe* protein |
| *PSD1* | 5.6 | 8.5 | Phosphatidylserine Decarboxylase 1 |
| *CAC2* | 5.5 | 4.3 | p60 subunit of the yeast CAF-I |
| *HSL1* | 5.5 | 2.3 | Negative regulator of swe1 kinase |
| *PRY3* | 5.5 | 3.2 | Similar to plant PR-1 class of pathogen proteins |
| *SCJ1* | 5.5 | 5.5 | dnaJ homolog |
| *SUC2* | 5.5 | 10.3 | Invertase (sucrose hydrolyzing enzyme) |
| YIL108W | 5.5 | 5.9 | Similarity to hypothetical *S. pombe* protein |
| YKR043C | 5.5 | 5.3 | Weak similarity to phosphoglycerate mutase |
| YMR166C | 5.5 | 6.3 | Similarity to mitochondrial carrier protein family |
| *EHT1* | 5.4 | 4.8 | Probable membrane receptor |
| *PAU6* | 5.4 | 8.4 | Member of the seripauperin protein\/gene family |
| *RVS167* | 5.4 | 5.2 | Involved in endocytosis |
| *SRB2 EX2* | 5.4 | 5.9 | RNA polymerase II holoenzyme\/mediator subunit |
| YLL064C | 5.4 | 6.8 | similarity to members of the Srp1/Tip1p family |
| YNL335W | 5.4 | 5.5 | Similarity to M.verrucaria cyanamide hydratase |
| *DED81* | 5.3 | 5.4 | Asparaginyl-tRNA synthetase |
| *FAS2* | 5.3 | 4.3 | Alpha subunit of fatty acid synthase |
| *GDH1* | 5.3 | 5.4 | NADP-specific glutamate dehydrogenase |
| *ISC1* | 5.3 | 5.7 | Putative neutral sphingomyelinase |
| *PMT4* | 5.3 | 5.4 | Dolichyl phosphate-D-mannose |
| *SRL1* | 5.3 | 5.2 | Suppressor of rad53 lethality |
| *SSA4* | 5.3 | 5 | Member of 70 kDa heat shock protein family |
| YHL021C | 5.3 | 6.2 | Similarity to Pseudomonas gamma-butyrobetaine |
| *ADE3* | 5.2 | 5.2 | Required for the biosynthesis of purines, thymidylate |
| *HXT16* | 5.2 | 3.1 | Hexose transporter |
| *NUD1* | 5.2 | 4.4 | Component of the spindle pole body |
| *PAN5* | 5.2 | 4.9 | Weak similarity to translational activator CBS2 |
| *SAC6 EX2* | 5.2 | 5.5 | Fibrim homolog (actin-filament bundling protein) |
| *STP22* | 5.2 | 5.9 | Ste pseudorevertant; required for vacuolar targeting |
| *DYN1* | 5.1 | 6.7 | Heavy chain of cytoplasmic dynein |
| *SIM1* | 5.1 | 5.7 | Involved in cell cycle regulation and aging |
| *XDJ1* | 5.1 | 6.3 | Homolog of *E. coli* DnaJ |

| | | | |
|---|---|---|---|
| YDR516C | 5.1 | 5.5 | Strong similarity to glucokinase |
| *GFD2* | 5 | 4.8 | Similarity to hypothetical protein YDR514c |
| *HAC1 EX2* | 5 | 8.7 | bZIP (basic-leucine zipper) protein |
| *HAC1 EX2* | 5 | 7.7 | bZIP (basic-leucine zipper) protein |
| *TAF1* | 5 | 5.2 | Similarity to *C. carbonum* toxin pump |
| *TOS3* | 5 | 6.1 | Ser/thr protein kinase |
| *TRM5* | 5 | 5.1 | tRNA modification enzyme |
| YBL109W | 5 | 5.7 | Similarity to subtelomeric encoded proteins |
| YJR030C | 5 | 3 | Similarity to hypothetical protein YJL181w |
| *AAC3* | 4.9 | 4.8 | Mitochondrial ADP/ATP translocator |
| *FPR4* | 4.9 | 5.1 | 60 kDa nuclear FK506 binding protein |
| *LYS1* | 4.9 | 4.7 | Saccharopine dehydrogenase |
| *PHO90* | 4.9 | 2.4 | Strong similarity to Pho87p |
| *PUS2* | 4.9 | 4.1 | Pseudouridine synthase 2 |
| *RET2* | 4.9 | 6.1 | Coatomer (COPI) complex delta subunit |
| *SQT1* | 4.9 | 4.9 | Involved in a late step of 60S ribosomal assembly |
| *ARP7* | 4.8 | 3.5 | Actin-related protein |
| *BAT1* | 4.8 | 4.8 | Branched-chain amino acid transaminase |
| *GAS1* | 4.8 | 5.1 | Cell surface glycoprotein 115-120 kDa |
| *KRS1* | 4.8 | 4.1 | Lysyl-tRNA synthetase |
| *PAN1* | 4.8 | 4 | Involved in actin organization and endocytosis |
| *PMT2* | 4.8 | 1.9 | Transfers mannosyl residues |
| YJR001W | 4.8 | 2.7 | Weak similarity to A.thaliana aminoacid permease |
| *EPT1 EX2* | 4.7 | 4.5 | sn-1,2-diacylglycerol ethanolamine |
| *FRDS* | 4.7 | 4.7 | Fumurate ReDuctase Soluble |
| *HEM12* | 4.7 | 4.8 | Uroporphyrinogen decarboxylase |
| *NOP1* | 4.7 | 4.3 | Nucleolar protein |
| *OPT1* | 4.7 | 2.9 | Oligopeptide transporter |
| *POR2* | 4.7 | 3.7 | Voltage dependent anion channel (YVDAC2) |
| *SPE1* | 4.7 | 2.2 | Rate limiting step of polyamine biosynthesis pathway |
| *TKL1* | 4.7 | 5.4 | Transketolase 1 |
| *TRP5* | 4.7 | 4.6 | Tryptophan synthetase |
| *TRR1* | 4.7 | 4.5 | Thioredoxin reductase |
| YGR111W | 4.7 | 6.2 | Weak similarity to mosquito carboxylesterase |
| *ASN1* | 4.6 | 5.3 | Asparagine synthetase |
| *CRZ1* | 4.6 | 5.3 | Calcineurin responsive zinc-finger |
| *MSK1* | 4.6 | 6 | Mitochondrial lysine-tRNA synthetase |
| *NRD1* | 4.6 | 4.9 | RNA recognition motif-containing protein |
| *SAM1* | 4.6 | 5.2 | S-adenosylmethionine synthetase |
| *SIT1* | 4.6 | 4.2 | Probably multidrug resistance protein |
| *WSC2* | 4.6 | 5.9 | Putative integral membrane protein |
| YEL017W | 4.6 | 4.5 | Hypothetical protein |
| YHR162W | 4.6 | 4.5 | Strong similarity to hypothetical protein YGR243w |
| YNL058C | 4.6 | 6.6 | Similarity to YIL117c |
| *ATS1* | 4.5 | 3 | Protein with similarity to human RCC1 protein |
| *FUN26* | 4.5 | 2.1 | Predicted membrane protein |

| | | | |
|---|---|---|---|
| *GNP1* | 4.5 | 6.6 | High-affinity glutamine permease |
| *HSP60* | 4.5 | 3.8 | Heat shock protein 60\; chaperonin protein |
| *HXT3* | 4.5 | 4.7 | High-affinity glucose transporter |
| *RPP1* | 4.5 | 2.7 | Protein subunit of nuclear ribonuclease P (RNase P) |
| *SUL2* | 4.5 | 5.8 | High affinity sulfate permease |
| *SVS1* | 4.5 | 5 | Serine and threonine rich protein. |
| YHR020W | 4.5 | 5 | Aminoacyl tRNA-synthetase |
| YLL012W | 4.5 | 4.6 | Similarity to triacylglycerol lipases |
| *HOG1* | 4.4 | 5.6 | Osmoregulation MAP kinase |
| *NAP1* | 4.4 | 3.7 | Nucleosome assembly protein I |
| *SUR7* | 4.4 | 5 | Multicopy suppressor of rvs167 mutation |
| *URA7* | 4.4 | 4.5 | CTP synthase, highly homologus to URA8 |
| *DIC1* | 4.3 | 4.7 | Mitochondrial dicarboxylate transport protein |
| *DIG1* | 4.3 | 4.7 | Down-regulator of Invasive Growth |
| *DPB2* | 4.3 | 4.9 | DNA polymerase epsilon, subunit B |
| *LAC1* | 4.3 | 4 | Longevity-assurance gene 1 cognate (LAG1 cognate) |
| *PAU3* | 4.3 | 5.5 | Member of the seripauperin protein/gene family |
| *PDR15* | 4.3 | 4.3 | Probable multidrug resistance transporter |
| *SCS3* | 4.3 | 4.6 | Required for inositol prototrophy |
| YCL044C | 4.3 | 5.4 | Hypothetical protein |
| YDR476C | 4.3 | 4.3 | Hypothetical protein |
| YHR032W | 4.3 | 6 | Ethionine resistance protein |
| YKL031W | 4.3 | 5.4 | Hypothetical protein |
| YPR118W | 4.3 | 5 | Sim. to *M. jannaschii* translation initiation factor |
| *BAR1* | 4.2 | 5.4 | Encodes a-cell barrier activity on alpha factor |
| *FRS1* | 4.2 | 4.2 | Phenylalanyl-tRNA synthetase, alpha subunit |
| *HAC1* | 4.2 | 6.7 | bZIP (basic-leucine zipper) protein |
| *PGD1* | 4.2 | 4.3 | Probable transcription factor, polyglutamine domain |
| *VRG4* | 4.2 | 5.3 | May regulate Golgi function and glycosylation |
| YMR215W | 4.2 | 4.5 | Similarity to GAS1 protein |
| *ZUO1* | 4.2 | 3.3 | Zuotin, putative Z-DNA binding protein |
| *DSK2* | 4.1 | 4.9 | Ubiquitin-like protein |
| *FCP1* | 4.1 | 3.6 | TFIIF interacting Component of CTD Phosphatase |
| *GCN20* | 4.1 | 3 | Member of ATP-binding cassette (ABC) family |
| *GSY1* | 4.1 | 5 | Glycogen synthase |
| *MCH5* | 4.1 | 4.3 | Similarity to human X-linked PEST-containing prot. |
| *NGL3* | 4.1 | 4.7 | Similarity to YMR285c |
| *PTC2* | 4.1 | 4.3 | Protein phosphatase type 2C |
| *TIM44* | 4.1 | 4 | 48.8 kDa protein involved in mitochondrial import |
| *TOM1* | 4.1 | 4.3 | Hect-domain-containing protein |
| *TPS3* | 4.1 | 4 | 115 kD regulatory subunit of trehalose-6-phosphate |
| YDR222W | 4.1 | 4.5 | Strong similarity to hypothetical protein YLR225c |
| YFR044C | 4.1 | 3.8 | Similarity to hypothetical protein YBR281c |
| YLR179C | 4.1 | 5.6 | Similarity to Tfs1p |
| YOL030W | 4.1 | 4.7 | Strong similarity to glycoprotein Gas1p |
| *ZWF1* | 4.1 | 5.1 | Glucose-6-phosphate dehydrogenase |

| | | | |
|---|---|---|---|
| *AAP1'* | 4 | 3.5 | Arginine/alanine aminopeptidase |
| *FAA1* | 4 | 5.4 | Long chain fatty acyl:CoA synthetase |
| *STE2* | 4 | 5.1 | Alpha-factor pheromone receptor |
| YER119C | 4 | 3.1 | Weak similarity to *E. herbicola* tyrosine permease |
| YGR068C | 4 | 4.8 | Weak similarity to Rod1p |

**Appendix Table 8.2.** Genes most significantly downregulated in *apc10Δ* cells. The results of two independent experiments are provided.

| Gene/ORF | Fold Δ1 | Fold Δ2 | Description |
|---|---|---|---|
| *ADH2* | -51.7 | -54.4 | Alcohol dehydrogenase II |
| *YRO2* | -45.6 | -33.5 | Homolog to HSP30 heat shock protein YRO1 |
| YCR101C | -37.4 | -29.3 | Strong similarity to *Y. lipolytica* GPR1 protein |
| *FBP1* | -36.6 | -36.5 | Fructose-1,6-bisphosphatase |
| *CLB5* | -24.8 | -2.1 | B-type cyclin |
| *ICS2* | -23.2 | -18 | Hypothetical protein |
| *PXA1* | -22 | -13.6 | Subunits of a peroxisomal ATP-binding cassette |
| *ATP20* | -21.3 | -17.2 | associated with mitochondrial ATP Synthase |
| YDL121C | -21 | -15.7 | Hypothetical protein |
| *PRM6* | -19.4 | -16.2 | Strong similarity to YJR054w |
| *ICL1* | -17.9 | -16.6 | Isocitrate lyase |
| *INH1* | -17.5 | -16.6 | ATPase inhibitor |
| YPL201C | -17.5 | -14.9 | Hypothetical protein |
| YDR384C | -16.4 | -17.1 | Strong similarity to *Y. lipolytica* GPR1 gene |
| *HXT5* | -16 | -16.8 | Hexose transporter |
| YLR16W | -15.7 | -12.7 | Strong similarity to Sdh4p |
| *COX6* | -14.9 | -13.8 | Subunit VI of cytochrome c oxidase |
| YOL038C | -14.4 | -12.2 | Identified by SAGE |
| YLR408C | -13.9 | -11.6 | Hypothetical protein |
| YMR11C | -13.9 | -11.6 | Strong similarity to succinate dehydrogenase |
| *PAI3* | -13.8 | -10.3 | Cytoplasmic inhibitor of proteinase Pep4p |
| *ATH1* | -13.7 | -10 | Null mutant: increased tolerance to dehydration |
| YER071C | -13.6 | -9.7 | Hypothetical protein |
| YDR27W | -13.5 | -8.2 | Weak similarity to YOR042w |
| *SPS100* | -13.3 | -6.6 | Sporulation-specific wall maturation protein |
| YIL015C-A | -13.1 | -11.1 | Strong similarity to hypothetical protein YIL102c |
| *OPY1* | -13.1 | -8.8 | Imparts Far- phenotype |
| *HNT3* | -13 | -11.3 | Hypothetical protein |
| *GRE1* | -12.9 | -10.8 | Induced by osmotic stress |
| YFR011C | -12.7 | -12.1 | Ochre suppressor tyr-tRNA |
| *ATP16* | -12.4 | -9.7 | ATP synthase delta subunit |
| *AQY1* | -12.2 | -12.6 | Aquaporin |
| *PHO89* | -12.1 | -8.7 | phosphate-repressible phosphate permease |
| YDL218W | -11.8 | -11.2 | Weak similarity to hypothetical protein YNR061c |
| YPR172W | -11.7 | -10.5 | Strong similarity to YLR456w |
| YOR044W | -11.4 | -10.7 | Weak similarity to YDR275w |
| DIB1 | -11.4 | -9.5 | *S. pombe* dim1+ in budding yeast |
| YLR211C | -11.1 | -7.6 | Hypothetical protein |
| *SME* | -11 | -8.7 | Required for pre-mRNA splicing |
| YGL121C | -10.9 | -8.3 | Hypothetical protein |

| | | | |
|---|---|---|---|
| *ARL3* | -10.9 | -5 | Strong similarity to ADP-ribosylation factors |
| *SPL2* | -10.7 | -11.2 | Suppressor of plc1-delta |
| *LSM8* | -10.4 | -17.6 | Sm-like protein |
| *TRX3* | -10.1 | -9.6 | Thioredoxin type II |
| *MLS1* | -10.1 | -8.1 | Carbon-catabolite sensitive malate synthase |
| *MDH2* | -10 | -9.2 | Cytosolic malate dehydrogenase |
| YOR304C-A | -10 | -8.8 | AIP3 binding protein |
| *APS1* | -9.9 | -7.7 | Clathrin-associated protein complex, small subunit |
| YDL157C | -9.7 | -10.2 | Hypothetical protein |
| *ELC1* | -9.7 | -7.7 | Elongin C transcription elongation factor |
| *ECM9* | -9.6 | -6.7 | ExtraCellular Mutant |
| *PCK1* | -9.5 | -9.8 | Phosphoenolpyruvate carboxylkinase |
| *SDH2* | -9.5 | -7.7 | Succinate de$H_2$ase (ubiquinone) iron-sulfur protein |
| *OLE1* | -9.5 | -7.2 | Delta-9-fatty acid desaturase |
| *PLP1* | -9.5 | -6.8 | GTPase inhibitor |
| *DAD2* | -9.4 | -9.9 | Weak similarity to *S. japonicum* paramyosin |
| *RKI1* | -9.3 | -7.2 | Ribose-5-phosphate ketol-isomerase |
| *SDC1* | -9.2 | -13 | Hypothetical protein |
| *DIP5* | -9.2 | -8.3 | Dicarboxylic amino acid permease |
| *ARO10* | -9.2 | -7.5 | Sim. to Pdc6p, Thi3p and pyruvate decarboxylases |
| *SRT1* | -9.1 | -8 | Similarity to YBR002c |
| *RPC10* | -9 | -8.3 | Subunit of RNA polymerase II |
| *COX4* | -9 | -8.2 | Subunit IV of cytochrome c oxidase |
| *RIP1* | -8.8 | -8 | mitochondrial cytochrome bc1 complex protein |
| *VPS20* | -8.8 | -5.7 | Similarity to SNF7 protein |
| *LOT6* | -8.8 | -4.9 | Weak similarity to *E. coli* hypothetical protein |
| YOR186W | -8.7 | -8.2 | Hypothetical protein |
| *SMD2* | -8.7 | -3.9 | U1 snRNP protein of the Sm class |
| *COX5A* | -8.6 | -7.4 | Cytochrome-c oxidase chain Va |
| YKL137W | -8.5 | -8.8 | Hypothetical protein |
| *VTI1* | -8.5 | -8.3 | v-SNARE that interacts with two t-SNARES |
| *RPL14B* | -8.5 | -7.2 | Ribosomal protein L14B |
| *FUI1* | -8.4 | -6 | Uridine permease |
| *ATP14* | -8.3 | -8.1 | ATP synthase subunit h |
| *SIP18* | -8.3 | -7 | Protein of unknown function |
| YLR036C | -8.3 | -6.5 | Similarity to YIL089w |
| *SPC1* | -8.2 | -11.9 | Homologue of the SPC12 subunit |
| YNR024W | -8.1 | -4.3 | Weak similarity to Rpc31p |
| *GAL10* | -8 | -7.2 | UDP-glucose 4-epimerase |
| YJL003W | -7.9 | -21.8 | Hypothetical protein |
| YBL028C | -7.9 | -7.8 | Involved in mating-type regulation |
| *MED8* | -7.9 | -6.3 | Stoichiometric member of mediator complex |
| YNL050C | -7.9 | -5.6 | Hypothetical protein |
| *QCR6* | -7.8 | -7 | Ubiquinol-cytochrome c oxidoreductase subunit |
| *LSM6* | -7.8 | -6.5 | Sm-like protein |
| *POP7* | -7.8 | -5.6 | integral subunit of RNase P |

| | | | |
|---|---|---|---|
| *LEE1* | -7.8 | -4.3 | Protein of unknown function |
| *MAK31* | -7.7 | -7.3 | MAK31 snRNP |
| *TEN1* | -7.7 | -6.7 | Similar to *A. aeolicus* adenylosuccinate synthetase |
| *REV3* | -7.7 | -6.2 | Translation initiation factor eIF1A |
| *TIF11* | -7.6 | -9 | DNA polymerase |
| YCR090C | -7.6 | -6.4 | Hypothetical protein |
| YCL056C | -7.5 | -6.4 | Hypothetical protein |
| *URA1* | -7.4 | -10.3 | Dihydroorotate dehydrogenase |
| *RIM1 EX1* | -7.4 | -7.1 | Required for mitochondrial DNA replication |
| *SPO73* | -7.4 | -6 | Hypothetical protein |
| YGR001C | -7.4 | -5.5 | Sim. to *C. elegans* hypothetical M142.5 protein |
| *UIP4* | -7.4 | -5.5 | Weak similarity to *Xenopus* protein xlgv7 |
| *RPS10A* | -7.4 | -5 | Ribosomal protein S10A |
| YPR151C | -7.4 | -4.8 | Weak similarity to YPL159c |
| *TIP20* | -7.4 | -4.3 | Transport protein that interacts with Sec20p |
| YHR022C | -7.3 | -6.5 | RAS-related protein |
| *TIM11* | -7.3 | -6.5 | Subunit e of mitochondrial F1F0-ATPase |
| YOL071W | -7.2 | -6.7 | Similarity to hypothetical S. pombe protein |
| *CBP4* | -7.2 | -5.8 | Rea for ubiquinol-cytochrome c reductase activity |
| YGR206W | -7.2 | -5.7 | Similarity to *Xenopus* transcription factor Oct-1.17 |
| YGL079W | -7.1 | -9.2 | Hypothetical protein |
| *TRI1* | -7.1 | -6.1 | Strong similarity to YOR295w |
| *MED11* | -6.9 | -6.7 | Hypothetical protein |
| *SEC66* | -6.9 | -5.9 | Glycoprotein complexed with Sec62p and Sec63p |
| *RPC11* | -6.9 | -5.7 | Sim. to S. *acidocaldarius* transc. elongation factor |
| YLR460C | -6.9 | -5.4 | Similarity to *C. carbonum* toxD protein |
| *YKE2* | -6.8 | -7.1 | Polypeptide 6 of a Yeast Non-native Actin Binding |
| *YMR31* | -6.7 | -6.8 | Mitochondrial ribosomal protein (precursor) |
| YDR286C | -6.7 | -5.5 | Hypothetical protein |
| YHL050C | -6.7 | -5.5 | Strong similarity to subtelomeric encoded proteins |
| *MAL33* | -6.7 | -4.3 | Maltose fermentation regulatory protein |
| *ERP4* | -6.7 | -4.2 | p24 protein involved in membrane trafficking |
| *YIL064W* | -6.6 | -7 | Weak similarity to fowlpox virus major core protein |
| *ECM13* | -6.6 | -6.4 | ExtraCellular Mutant |
| YGL146C | -6.6 | -6 | Hypothetical protein |
| *VPS28* | -6.6 | -4.5 | Soluble, hydrophilic protein involved in transport |
| YER121W | -6.5 | -5.6 | Hypothetical protein |
| YOR289W | -6.5 | -5.5 | Similarity to *C.elegans* hypothetical protein |
| YDR031W | -6.5 | -5.3 | Hypothetical protein |
| YBR022W | -6.5 | -4.7 | Hypothetical protein |
| YKL061W | -6.4 | -12.7 | Hypothetical protein |
| *ACS1* | -6.4 | -11 | Inducible acetyl-coenzyme A synthetase |
| BIO4 | -6.4 | -10.2 | Dethiobiotin synthetase |
| YOL146W | -6.4 | -6.4 | Hypothetical protein |
| *PHM6* | -6.4 | -5.4 | Hypothetical protein |
| YPL071C | -6.3 | -5.9 | Hypothetical protein |

| | | | |
|---|---|---|---|
| *FRQ1* | -6.3 | -5.6 | Strong similarity to human BDR-1 protein |
| *HUB1* | -6.3 | -5.3 | Similar to ubiquitin-like protein 8 of *A. thaliana* |
| *DCP1* | -6.3 | -3.7 | Decapping protein involved in mRNA degradation |
| *ACH1* | -6.3 | -3.6 | Acetyl CoA hydrolase |
| *NAT3* | -6.2 | -5.5 | Similarity to N-acetyltransferases |
| *RSM27* | -6.2 | -5 | Strong similarity to hypothetical *S. pombe* protein |
| YHR081W | -6.2 | -3.8 | Weak similarity to human C1D protein |
| *SPC19* | -6.1 | -6.5 | Component of spindle pole |
| *MET28* | -6.1 | -6.5 | Transcriptional activator of sulfur aa metabolism |
| YML030W | -6.1 | -6.2 | Hypothetical protein |
| *SEN15* | -6.1 | -6.1 | 15kDa subunit of the tRNA splicing endonuclease |
| YDR034W | -6.1 | -6.1 | Identified by SAGE expression analysis |
| YIL024C | -6.1 | -6 | Hypothetical protein |
| *ATP5* | -6.1 | -5.9 | ATP synthase subunit |
| *ATP17* | -6.1 | -5.9 | ATP synthase subunit f |
| *MGT1* | -6.1 | -5.8 | 6-O-methylguanine-DNA methylase |
| YCR082W | -6.1 | -4.9 | Weak similarity to Rbk1p |
| *SNO1* | -6.1 | -4.8 | SNZ1 proximal ORF, stationary phase induced gene |
| *NCA3* | -6 | -14.9 | Regulates proper expression of subunit 6 (Atp6p) |
| *COX9* | -6 | -6.2 | Subunit VIIa of cytochrome c oxidase |
| YPR100W | -6 | -5.5 | Similarity to *C. elegans* hypothetical protein |
| *ARC18* | -6 | -5.4 | Arp2/3 Complex Subunit |
| *ATP3* | -6 | -5.4 | Gamma subunit of mitochondrial ATP synthase |
| YGR207C | -6 | -5.2 | Electron-transferring flavoprotein, beta chain |
| *SRN2* | -6 | -5.2 | Suppressor of rna1-1 mutation |
| *COX12* | -5.9 | -5.4 | Subunit VIb of cytochrome c oxidase |
| YGR146C | -5.9 | -4.9 | Hypothetical protein |
| *RDI1* | -5.9 | -4.7 | Rho GDP dissociation inhibitor |
| *PRE3* | -5.8 | -8.7 | Subunit of 20S proteasome |
| *PRP38* | -5.8 | -7 | RNA splicing factor |
| *SRB6* | -5.8 | -6.6 | Transcription factor, part of Srb\/Mediator complex |
| *QCR10* | -5.8 | -6.6 | ubiqunol-cytochrome c oxidoreductase complex |
| *SYF2* | -5.8 | -6.3 | Synthetic lethal with cdc40 |
| *RNY1* | -5.8 | -5.5 | Similarity to ribonucleases |
| *PET18* | -5.8 | -5 | Transcription regulator |
| YDR179C | -5.8 | -4.9 | Hypothetical protein |
| *FYV6* | -5.8 | -4.8 | required for Yeast Viability on toxin exposure |
| YGR110W | -5.8 | -4.1 | Weak similarity to YLR099c and YDR125c |
| *TAF19* | -5.8 | -3.9 | TFIID subunit |
| YIL040W | -5.7 | -6 | Weak similarity to *T. brucei* NADH dehydrogenase |
| YER030W | -5.7 | -5.9 | Similarity to mouse nucleolin |
| YLR262C-A | -5.7 | -5.4 | Strong similarity to a protein from *C. elegans* |
| *IMG2* | -5.7 | -4.9 | Integrity of Mitochondrial Genome 2 |
| *TPT1* | -5.7 | -4.4 | tRNA 2 -phosphotransferase |
| *BET5* | -5.7 | -4.1 | Bet5p\/18kD component of TRAPP |
| *EFR3* | -5.7 | -4 | Unable to sporulate or grow on acetate medium. |

| | | | |
|---|---|---|---|
| *ARV1* | -5.7 | -3.7 | Weak sim. to *C. elegans* protein and Nup120p |
| *ATP15* | -5.6 | -6.3 | Nuclear gene for ATP synthase epsilon subunit |
| YLR243W | -5.6 | -3.9 | Strong similarity to YOR262w |
| *REV7* | -5.5 | -6.3 | Subunit of DNA polymerase-zeta (Pol-zeta) |
| YOL132W | -5.5 | -6.2 | Sim. to glycophospholipid-anchored glycoprotein |
| *PUT4* | -5.5 | -5.6 | Putative proline-specific permease |
| *KRE27* | -5.5 | -5.3 | Toxin Resitance |
| YBR269C | -5.5 | -4.9 | Hypothetical protein |
| *OST4* | -5.5 | -4.8 | Weak similarity to mucin |
| *MRPL23* | -5.5 | -4.7 | Ribosomal protein of the large subunit, mitochon. |
| *ATP18* | -5.5 | -4.5 | Protein associated to the ATP synthase |
| *POP6* | -5.4 | -5.8 | Integral subunit of RNaseP |
| *RPL14B* | -5.4 | -5.8 | Ribosomal protein L14B |
| *VMA21* | -5.4 | -5.3 | Protein involved in vacuolar H-ATPase assembly |
| YBR230C | -5.4 | -5.1 | Hypothetical protein |
| *MRPL39* | -5.4 | -4.8 | Mitochondrial ribosomal protein MRPL39 (YmL39) |
| *EDS1* | -5.4 | -4.1 | Probable regulatory Zn-finger protein |
| *LSM2 EX2* | -5.4 | -4 | snRNA-associated protein of the Sm class |
| *GLC7 EX1* | -5.4 | -2.9 | Protein phosphatase type I |
| *JEN1* | -5.3 | -7.3 | Carboxylic acid transporter protein homolog |
| *NDT80* | -5.3 | -7.1 | Meiosis-specific gene |
| *LTP1* | -5.3 | -4.9 | 18-kDa phosphotyrosine phosphatase |
| YPL170W | -5.3 | -4.8 | Similarity to *C. elegans* LIM homeobox protein |
| *BSD2* | -5.3 | -3.8 | Metal homeostasis protein |
| *YIM1* | -5.3 | -2.9 | Mitochondrial inner membrane protease |
| *PDR10* | -5.2 | -4.8 | Putative ABC transporter highly similar to Pdr5p |
| *HSH49* | -5.2 | -4.4 | Homolog of mammalian splicing factor/U2 snRNP |
| YOR284W | -5.2 | -4.4 | Similarity to *M. jannaschii* hypothetical protein |
| *YPT7* | -5.2 | -3.7 | GTP-binding protein of the rab family |
| *ULP1* | -5.2 | -3.5 | Weak similarity to Smt4p |
| *GLC7* | -5.2 | -2.9 | Protein phosphatase type I |
| *RSC58* | -5.1 | -6.9 | Hypothetical protein |
| *ATX1* | -5.1 | -5 | Antioxidant protein and metal homeostasis factor |
| *CBP6* | -5.1 | -4.3 | Translational activator of COB mRNA |
| YIL055C | -5.1 | -4.1 | Hypothetical protein |
| *MCM21* | -5.1 | -3.9 | Involved in minichromosome maintenance |
| YLR456W | -5 | -5.8 | Strong similarity to YPR172w |
| YDR071C | -5 | -5.1 | Sim. to *O. aries* arylalkylamine N-acetyltransferase |
| *APG12* | -5 | -4.2 | Autophagy |
| *RPS7B EX2* | -5 | -4.2 | Ribosomal protein S7B (rp30) |
| *SPC3* | -5 | -4.2 | Signal peptidase subunit |
| *APG10* | -5 | -3.9 | Hypothetical protein |
| *RER1* | -5 | -3.8 | Involved in retention of membrane proteins |
| *TLG1* | -5 | -3.2 | tSNARE that affects a Late Golgi compartment |
| *SNO2* | -4.9 | -5.3 | SNZ2 proximal stationary phase induced gene |
| *CTA1* | -4.9 | -5.1 | Catalase A |

| | | | |
|---|---|---|---|
| *RPS7A* | -4.9 | -4.9 | Ribosomal protein S7A (rp30) |
| YDL219W | -4.9 | -4.9 | Strong similarity to *S. equisimilis* protein |
| YGL232W | -4.9 | -4.7 | Sim. to *P. falciparum* dihydropteroate synthase |
| YHR140W | -4.9 | -4.2 | Hypothetical protein |
| *NMD4* | -4.9 | -3.8 | Putative Upf1p-interacting protein |
| *YAR1* | -4.9 | -3.7 | 2 ANK repeat motifs and an acidic C terminus |
| *CKB2* | -4.9 | -3.5 | Casein kinase II, beta subunit |
| YOR093C | -4.9 | -3.5 | Similarity to *S. pombe* hypothetical protein |
| *SPC2* | -4.8 | -6.7 | Subunit of signal peptidase complex |
| YLR297W | -4.8 | -6.4 | Weak similarity to *Vibrio vulnificus* VvpC protein |
| *TIM9* | -4.8 | -4.8 | Translocase in inner membrane of mitochondria |
| *YAT2* | -4.8 | -4.5 | Similarity to carnitine O-acetyltransferase Yat1p |
| YKR074W | -4.8 | -4.3 | Strong similarity to hypothetical *S. pombe* protein |
| *GRX4* | -4.8 | -3.7 | Similarity to *Legionella* glutaredoxin-like protein |
| YPL257W | -4.8 | -3.5 | Weak similarity to YIL029c |
| YGL136C | -4.8 | -3.1 | Weak similarity to *E. coli* ftsJ protein |
| YOR253W | -4.7 | -8.1 | Hypothetical protein |
| *RFA3* | -4.7 | -6.2 | Subunit 3 of replication factor-A |
| YHR126C | -4.7 | -4.8 | Hypothetical protein |
| *DID2* | -4.7 | -4.6 | RAD52 Inhibitor (Fifty Two Inhibitor) |
| *YAT1* | -4.7 | -4.5 | Outer carnitine acetyltransferase, mitochondrial |
| YDR357C | -4.7 | -4.5 | Hypothetical protein |
| YNL144C | -4.7 | -4.2 | Similarity to YHR131c |
| *HMLALPHA2* | -4.7 | -4 | Mating type protein alpha-2 |
| *MRP2* | -4.7 | -3.9 | 14 kDa mitochondrial ribosomal protein |
| *ASI1* | -4.7 | -3.9 | Amino acid Sensor-Independent (ASI) gene |
| YBR194W | -4.7 | -3.6 | Hypothetical protein |
| *SKN1* | -4.7 | -3.1 | predicted type II membrane protein |
| YGL010W | -4.6 | -5.3 | Similarity to hypothetical S. pombe protein |
| *RUB1 EX1* | -4.6 | -4.9 | Ubiquitin-like protein |
| YML133C | -4.6 | -4.5 | Hypothetical protein Y .2 |
| YLL049W | -4.6 | -4.4 | Hypothetical protein |
| *SRB7* | -4.6 | -4.4 | RNA polymerase II holoenzyme component |
| *COX8* | -4.6 | -4.2 | Cytochrome-c oxidase chain VIII |
| *RIM1 EX2* | -4.6 | -4.2 | Required for mitochondrial DNA replication |
| *QRI5* | -4.6 | -4.1 | Protein of unknown function |
| *TTR1* | -4.6 | -4 | Glutaredoxin (thioltransferase) |
| *GPM2* | -4.6 | -3.9 | Similar to GPM1 (phosphoglycerate mutase) |
| *ECM33* | -4.6 | -3.7 | Homolog to sporulation specific protein SPS2 |
| *SMD3* | -4.6 | -3.5 | Encodes a core snRNP protein |
| *ECM15* | -4.6 | -3.3 | ExtraCellular Mutant |
| YDL199C | -4.6 | -3.3 | Similarity to sugar transporter proteins |
| *URM1* | -4.5 | -4.9 | Hypothetical protein |
| YDR163W | -4.5 | -4.7 | Weak similarity to *S. pombe* hypothetical protein |
| *LSM5* | -4.5 | -4.3 | Sm-like protein |
| *MLC2* | -4.5 | -4.2 | Similarity to calmodulin |

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|---|---|---|---|
| YLR021W | -4.5 | -3.9 | Hypothetical protein |
| *RPS23B* | -4.5 | -3.9 | Ribosomal protein S23B (S28B) (rp37) (YS14) |
| *GDH2* | -4.5 | -3.8 | NAD-dependent glutamate dehydrogenase |
| YGR024C | -4.5 | -3.7 | sim. to *M. thermoautotrophicum* protein MTH972 |
| *NEJ1* | -4.5 | -3.6 | Hypothetical protein |
| *STE20* | -4.5 | -3.2 | Serine/threonine protein kinase |
| YDR482C | -4.4 | -7.1 | Hypothetical protein |
| YOR252W | -4.4 | -4.5 | Hypothetical protein |
| *IDP2* | -4.4 | -4 | Form of NADP-dependent isocitrate de$H_2$ase |
| *RPL12B* | -4.4 | -4 | Ribosomal protein L12B (L15B) (YL23) |
| *DPB4* | -4.4 | -4 | Weak similarity to YNC2beta protein |
| *YKU70* | -4.4 | -3.8 | DNA binding protein |
| YGL226W | -4.4 | -3.6 | Similarity to *N. crassa* cytochrome-c oxidase |
| *DAL80* | -4.4 | -3.4 | Negative regulator of nitrogen catabolic genes |
| *UBC8* | -4.4 | -3.3 | Ubiquitin-conjugating enzyme |
| *OST2* | -4.4 | -2.8 | Epsilon subunit of oligosaccharyltransferase |
| *APG9* | -4.3 | -5.1 | Null mutant is viable |
| *SEM1* | -4.3 | -5 | Homolog of DSS1 |
| YIL057C | -4.3 | -5 | Strong similarity to YER067w |
| *EDC2* | -4.3 | -4.6 | Enhancer of mRNA Decapping |
| YDR070C | -4.3 | -4.5 | Hypothetical protein |
| YGL242C | -4.3 | -4.5 | Weak similarity to *Drosophila* ANK protein |
| *KKQ8* | -4.3 | -4.2 | Probable serine/threonine-specific protein kinase |
| *YTH1* | -4.3 | -4 | Yeast 30kDa Homologue |
| YER048W-A | -4.3 | -3.7 | Putative ORF identified by SAGE |
| YEL057C | -4.3 | -3.5 | Hypothetical protein |
| *RPS28B* | -4.3 | -3.5 | Ribosomal protein S28B (S33B) (YS27) |
| *MRPL17* | -4.3 | -3.4 | Ribosomal protein of the large subunit; mitochon. |
| YLR065C | -4.3 | -3.4 | Hypothetical protein |
| YOR164C | -4.3 | -3.4 | Hypothetical protein |
| YPL107W | -4.3 | -3.3 | Hypothetical protein |
| *OST6* | -4.3 | -3.1 | Putative subunit of N-oligosaccharyltransferase |
| *MET4* | -4.3 | -2.6 | Member of the leucine zipper family of TFs |
| *ATP7* | -4.2 | -6.8 | ATP synthase d subunit |
| *ASI1* | -4.2 | -4.1 | Similarity to YNL008c |
| *RPL36A* | -4.2 | -4 | Ribosomal protein L36A (L39) (YL39) |
| *SMM1* | -4.2 | -4 | Suppressor of Mitochondrial Mutation |
| YBL107C | -4.2 | -3.9 | Hypothetical protein |
| *MRPL15* | -4.2 | -3.5 | Mitochondrial ribosomal protein MRPL15 |
| YGR052W | -4.2 | -3.5 | Similarity to ser/thr protein kinases |
| *IAH1* | -4.2 | -3.3 | Isoamyl acetate hydrolytic enzyme |
| *BOP2* | -4.2 | -3.2 | Bypass of PAM1 |
| *VMA8* | -4.2 | -3.1 | Vacuolar H-ATPase subunit |
| *RFC5* | -4.2 | -2.9 | Subunit 5 of Replication Factor C |
| *CYC1* | -4.1 | -20.7 | Iso-1-cytochrome c |
| YJL161W | -4.1 | -4.9 | Hypothetical protein |

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|---|---|---|---|
| *NFU1* | -4.1 | -4.8 | NifU-like protein B |
| YGL080W | -4.1 | -4.8 | Strong similarity to *C. elegans* R07E5.13 protein |
| *SNL1* | -4.1 | -4.6 | 18.3 kD integral membrane protein |
| YDR339C | -4.1 | -4.4 | Weak similarity to hypothetical protein YOR004w |
| *OST4* | -4.1 | -4.3 | 3.6-kDa protein, probably membrane-located |
| *MRPL44* | -4.1 | -3.9 | Mitochondrial ribosomal protein MRPL44 |
| *YNG2* | -4.1 | -3.8 | NuBbiN |
| *NCE103* | -4.1 | -3.6 | Involved in secretion of proteins |
| YHR121W | -4.1 | -3.6 | Weak similarity to C.elegans hypothetical protein |
| *ERV1 EX1* | -4.1 | -3.3 | Protein essential for mitochondrial biogenesis |
| *GTR1* | -4.1 | -3.2 | Putative small GTPase |
| *TPM2* | -4.1 | -3 | Tropomyosin isoform 2 |
| YGR081C | -4.1 | -3 | similarity to mammalian myosin heavy chain |
| YIR044C | -4.1 | -2.6 | Putative pseudogene |
| *MNT2* | -4.1 | -2.5 | MaNnosylTransferase |
| YLR051C | -4 | -7.5 | Similarity to human acidic 82 kDa protein |
| *CTF8* | -4 | -6.4 | Putative kinetochore protein |
| YCR085W | -4 | -5.7 | Hypothetical protein |
| *GIM4* | -4 | -4.3 | Putative homolog of bovine prefoldin, a chaperone |
| YPL064C | -4 | -3.9 | Similarity to peptidyl-prolyl cis-trans isomerase |
| *ARL1* | -4 | -3.8 | ADP-ribosylation factor-like protein 1 |
| *UBC5 EX2* | -4 | -3.5 | Ubiquitin-conjugating enzyme |
| YNL211C | -4 | -3.4 | Hypothetical protein |
| YNR022C | -4 | -3.2 | Weak similarity to protein phosphatases |
| *UBC12 EX2* | -4 | -3.1 | Ubiquitin-conjugating enzyme |
| YGR046W | -4 | -3 | Hypothetical protein |
| *MRPL25* | -4 | -2.9 | Mitochondrial ribosomal protein MRPL25 |
| *RRN10* | -4 | -2.9 | Upstream activation factor subunit |
| *BLM3* | -4 | -2.3 | Protects cell against bleomycin damage |
| YOL008W | -4 | -2.2 | Hypothetical protein |